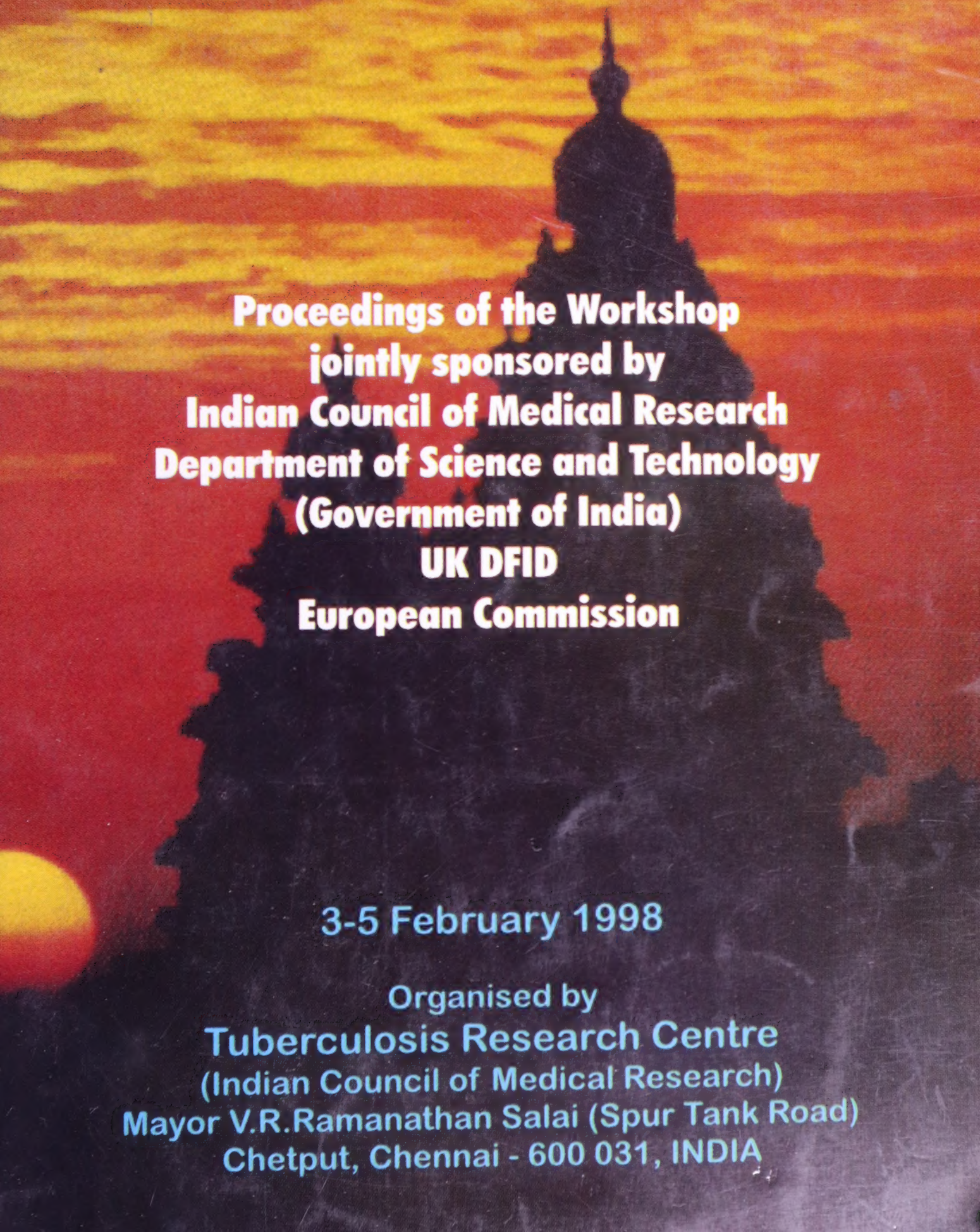
TUBERCULOSIS RESEARCH : INTO THE 21st CENTURY



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Organised by
Tuberculosis Research Centre
(Indian Council of Medical Research)
Mayor V.R.Ramanathan Salai (Spur Tank Road)
Chetput, Chennai - 600 031, INDIA

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PREFACE

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Tuberculosis Research in 21st Century

About two billion people carry living tubercle bacilli in them-potential time-bombs which can go off at any time. Of these, in about 200 million the bomb may go off, leading to the development of tuberculosis disease over the next few decades, the rest of the people will live with the bacilli in peaceful coexistence. However, with a TB-friendly human immuno-deficiency virus around, we may see many more people developing tuberculosis, with greater risk of transmission of the bacillus to others, and maintaining the pool of infection at the level of a third of the world population. Five decades of TB control programmes, using potentially efficacious drugs, have failed to cause reduction in the incidence and prevalence of infection and of disease in most parts of the world.

To effect a perceptible reduction in incidence, there has to be a substantial reduction in the pool of infection and in transmission - goals which can be achieved through a well-managed tuberculosis control strategy. DOTS - Directly Observed Treatment Short-Course promises to achieve these goals and to go beyond. Apart from curing patients in the shortest period, DOTS reduces the risk of emergence of drug resistance. Hope for TB control lies in all countries adopting, owning and implementing DOTS extensively.

The DOTS strategy itself is not static. It needs to be adopted according to local situations, and with use of newly emerging scientific data. Science has much to offer to DOTS through evolving better tools in TB control through basic research - tools that help us to more clearly understand the host-parasite interaction and to evolve better diagnostic procedures and better tests for detection of infection and disease, to develop more effective vaccines and newer drugs that selectively target the bacillus. Science can also help us to evolve appropriate control strategies, making optimal use of available tools and resources.

India has been a leader in providing scientific data to the world through research in several areas, including epidemiology, clinical management, pharmaco-kinetics and operational research in tuberculosis control. The global TB control strategy today has evolved through research conducted over decades in countries spread all over the world. It has truly been a collective effort. Hopefully, this spirit of partnership and collaboration can be sustained in future years, resisting pressures from commercial interests keen on exploitation through patents. With the spectre of AIDS, the pace of TB control needs to

be considerably speeded up. Scientists must coordinate their studies so that they complement each other, make optimum use of resources and information, avoid duplication of efforts and achieve speedy results.

The scientific community and responsible international aid agencies are fully aware that with a concerted global control strategy we will soon see a steady decline in the TB problem. With spirited and collective contributions from the scientists, the health administrators, the healthcare providers and the international aid agencies, we may be entering the next century with a firm determination to eliminate tuberculosis in the first quarter.

This meeting of research scientists organised through the auspices of the European Union, the British Department For International Development, the Indian Council of Medical Research and the Department of Science and Technology, has provided an opportunity for scientists from India and abroad to interact with each other, exchange scientific information and to forge partnership and to collaborate with each other.

We must spare no efforts to achieve our goal of elimination of tuberculosis. The senseless death of 3-4 million people from tuberculosis each year must be prevented. Preventable they are, and if the global TB strategy is effectively implemented, the number of deaths would be substantially reduced. Let us all strive to make this dream a reality.

SCOPE OF THE WORKSHOP

Tuberculosis is a curable and controllable disease. The paradox is that in 1993, the World Health Organisation declared Tuberculosis as a Global Emergency. It is estimated that between now and 2020 nearly one billion more people will be newly infected, 200 million people will get sick and 17 million will die from tuberculosis, if control programmes are not implemented properly. Tuberculosis kills more youth and adults than any other disease in the world.

In India, it is estimated that there are 3.5 million sputum positive cases and approximately 1.5 million cases are detected and put on treatment every year. Approximately 0.5 million deaths from tuberculosis occur every year. HIV is accelerating the spread of tuberculosis. Persons infected with *M.tuberculosis* who are also HIV infected have at least 50% life time risk of developing tuberculosis, with an annual risk of developing disease of approximately 7-10%, which is many times higher than that of HIV negative patients. Poorly managed TB programmes are responsible for the increase in multidrug resistance. HIV and MDR-TB are increasing the tuberculosis burden of the world.

The success of TB control programmes depends on the successful completion of treatment by patients. Realising the importance of this principle the Revised National TB Programme of Govt. of India emphasizes the necessity of DOTS strategy for tuberculosis control. DOTS can certainly provide very high cure rates for most patients who are reached by the control programme. However, researchers all over the world are now convinced that a good programme should also have a powerful research component backing it up which will continuously provide better tools for diagnosis, treatment and monitoring. A good example of this need is the case of reemergence of tuberculosis in the developed countries as a consequence of the HIV epidemic. These countries had good programmes in place using powerful chemotherapeutic agents and BCG prophylaxis and adequate surveillance. Yet there was a resurgence of tuberculosis even in that setting. Similarly, since there has been no new drug development in this field for the past two decades health personnel were left defenceless in the management of disease caused by MDRTB organisms. These events clearly indicate the need to generate more research tools in the future for tuberculosis control and elimination.

This workshop was designed to meet that need. Taking cognizance of the major scientific breakthroughs in tuberculosis research over the past years, this workshop was organised around various topic of importance in the field. Special attention has been given to modern molecular genetics, the tuberculosis, genome project, new drug and vaccine development and cellular immunology.

The workshop brought together leading researchers in this filed from Europe and their Indian counterparts. It provided an opportunity for the scientists to present their concepts and data during their lectures which were followed by critical discussions. There was an unanimous opinion that DOTS is the best help that is available today, but it alone is insufficient to reduce tuberculosis incidence appreciably on a Global scale. The highlight of the workshop were the recommendations for future research made by the participantsand an agreement to share knowledge and experience with a common goal of controlling the most dreaded disease of mankind.

M. J. Colston

P. R. Narayanan

"REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAMME (RNTCP)

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A burden of disease study in India undertaken for 1993 World Development Report revealed that the burden of tuberculosis is 11 times more than malaria, 6 times more than tropical cluster of diseases and 2½ times more than AIDS. Tuberculosis incidence peaks in the 15-45 years age group, which is the most economically productive part of life. In 1993 tuberculosis was responsible for 10% of all disability adjusted life years (DALYS) for males and just over 5% for females. Tuberculosis is also a major cause of death amongst economically active population. It kills more adults than any other infection.

Sir Robert Koch announced the discovery of *Mycobacterium tuberculosis* on 24th March 1882, potent anti-TB drugs were discovered in 1950s, yet tuberculosis continues to be the most common serious infectious disease in the world. Globally about 8 million new cases occur each year. 3 million people died of tuberculosis in 1995 surpassing the worst years of TB epidemic in 1900s when 2.1 million people died of tuberculosis every year. Nearly 2 out of every 5 TB cases in the world are found in India. 15 lakh new cases of TB report to the programme every year. As estimated 5 lakhs people in India die every year of tuberculosis-more than 1000 every day a patient a minute. The countries of SEAR account for 42% of the global burden of reported tuberculosis cases. India accounts for 2/3rd of the SEAR burden of TB.

To tackle the problem of tuberculosis, Government of India launched a National Tuberculosis Control Programme in 1962. The programme built up a good infrastructure by establishing 446 District TB Centres, 330 TB Clinics and over 47,600 beds. However, inspite of best efforts, the programme did not make a significant dent on the problem of Tuberculosis. The emphasis had been on case detection rather than cure. The diagnosis of infectious cases took aback seat in the programme. The treatment completion rate has been to the tune of only 30%. With this background National TB Control Programme was reviewed in 1992 by a group of experts representing the Govt. of India, World Health Organisation and Swedish International Development Agency.

On the recommendations of this expert committee, a revised strategy to control TB was formulated which is known as DOTS strategy or RNTCP. There are 5 main components of DOTS strategy:-

- Political will
- Diagnosis by microscopy of patients presenting to health facilities
- Short course chemotherapy by direct observation
- Regular supply of good quality drugs
- Intensive supervision and monitoring

This strategy was pilot tested in 1993 in a population of 2.35 million. Encouraged by the results, the pilot study was extended to cover 13.85 million population in 13 States/UTs of the country. In these areas, diagnostic practices improved with effective use of quality sputum microscopy, and cure rates more than doubled as compared to those achieved with traditional treatment. Because of these encouraging results, it was considered appropriate to expand the coverage area. A soft loan of US $ 142.4 million was successfully negotiated with World Bank. RNTCP was formally launched in India on 26th March, 1997 initially in 102 districts covering a population of 271.2 million in a phased manner. It is expected that from World TB Day in 1998 service delivery will commence in 39 districts covering a population of 124.7 million. The goal of RNTCP is to cure at least 85% of new sputum positive patients detected. Having achieved the desired cure rates, emphasis will be laid on detecting at least 70% of such cases.

The key to the success of the DOTS strategy is that it places the responsibility for curing the TB patients on the health workers - not the patients. Patients with tuberculosis are the VIPs of the programme. RNTCP has been remarkably successful. In a population of more than 200 lakhs in 13 states throughout the country, the quality of diagnosis is dramatically better than that of the previous programme or of private practitioners. The ratio between sputum positive and negative cases is almost 1:1 which by any technical standards is the ideal case detection paradigm. Nearly 8 out of 10 patients diagnosed in the programme since 1993 were cured; this cure rate is more than double that of the previous programme.

Patient non-adherence to treatment, even if medications are available, is a serious problem reducing the success of tuberculosis control measures and facilitating the spread of drug resistance. There are many causes for patient non-adherence, including poor interpersonal communication skills and inaccessibility of treatment facilities. The only reliable and proven method of ensuring adherence to treatment is in a well-functioning programme of directly observed treatment (DOT), in which a trained individual who is not a family member watches and assists as TB patients swallow their pills. DOT is arranged as conveniently to the patient as possible, relying on strengths and resources which exist in each community (e.g., multi-purpose workers, Anganwadi workers, trained Dais, Panchayat leaders, religious leaders, etc.).

The Government of India has significantly increased the national budget for TB Control. The RNTCP will be implemented in a phased manner in a population of nearly 300 million in the next three years. At the same time, the rest of the country will be prepared for RNTCP implementation by receiving updated technical material, diagnostic equipment, uninterrupted supply of drugs, and by implementing the RNTCP registration system. It is hoped that the RNTCP will be implemented nationally as soon as operationally feasible.

Experts caution that DOTS must not be implemented too rapidly. The experience in the past 4 years in India, which matches that of many countries, is that phased expansion is critical. Trying to expand too fast can result in a poor programme which can actually worsen the prospects for TB Control by increasing drug resistance.

Effective implementation of DOTS will save hundreds of thousands of lives in India. DOTS has been deemed one of the most cost-effective health interventions. Each life saved represents a child, mother, or father who will go on to live a productive, TB-free, longer life. Every patient who is cured stops spreading TB. Working together to implement DOTS, we can win the age-old battle against TB.

MDR-TB strains of the bacterium which are resistant to the two most active anti-TB drugs, Isoniazid and Rifampicin are a potential threat to successful treatment of TB. Treatment of MDR-TB is extremely expensive, toxic, arduous, and often unsuccessful. DOTS has been proven to prevent the emergence of MDR-TB, and also to reverse MDR-TB where it has emerged. MDR-TB is a tragedy for individual patients and a symptom of poor programme performance. The only way to confront this challenge is to improve the treatment programme and implement DOTS as rapidly as possible. A poorly performing programme will create drug-resistant cases at a faster rate than these cases can be cured, even if unlimited resources are available. There is need to establish a community based drug resistance surveillance system with in-built quality assurance programme.

While the size of the HIV epidemic in India is presently not known, it is clear that HIV will worsen the TB epidemic. The Human Immunodeficiency virus breaks down the immune system and makes patient highly susceptible to TB; these patients can then spread TB to other people. In some countries, the HIV epidemic has doubled or tripled TB cases. Fortunately, DOTS is as effective among HIV-infected TB patients as among those who are HIV negative. Even among HIV-infected TB patients, DOTS cures patients and results in longer, healthier lives.

Over half of the tuberculosis patients are managed by general practitioners who employ several regimens, varying in composition, duration of treatment and levels of efficacy. There is urgent need for harnessing private practitioners & non-governmental organizations in adopting DOTS for the treatment of tuberculosis.

WHO member countries have set global targets for 2000 A.D. to successfully treat 85% of sputum positive cases and detect 70% of such cases. So far, 100 countries have introduced DOTS strategy, however only 15% of all infectious cases are now being treated with DOTS. By the year 2000, 130 out of 214 countries would be achieving the global targets. DOTS strategy in India only covers 2% of its population at the present moment.

It is clear from the consideration of the preparatory steps needed for the implementation of the 5 components of DOTS that countries of the size & diversity such as of India cannot implement DOTS countrywide overnight - notwithstanding the political will and commitment and allocation of funds. It requires a lot of infrastructural strengthening, recruitment & training of staff and above all ensuring intensive supervision and monitoring. The feasibility of successful implementation of RNTCP in India has been demonstrated and hopefully by 2010 the total country would be reaping the benefits of the revised strategy of tuberculosis control programme.

This workshop on "Tuberculosis Research into the 21st century" is very timely. The topics chosen in the workshop encompass all facets of disease namely epidemiology, RNTCP, immunology, vaccine and drug development, immunodiagnosis and molecular biology - which will help in conduction of meaningful operational research in the forthcoming years resulting in programme improvement eventually.

Proceedings of the Workshop

HLA TRANSGENIC MICE AND SYNTHETIC PEPTIDE LIBRARIES AS NOVEL TOOLS FOR THE DEFINITION OF HLA RESTRICTED, T CELL STIMULATING ANTIGENS IN INFECTIOUS DISEASES.

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Introduction

Mycobacterial pathogens are major causes of morbidity and mortality worldwide. A third of the World population is infected with *M.tuberculosis*. Each year 8-10 million new active pulmonary tuberculosis cases arise and 3 million patients die of the disease, more than of any other single infectious organism. The incidence of tuberculosis is rising and virulent multidrug resistant strains are emerging.

The widely used tuberculosis vaccine strains, *M.bovis* BCG, continue to be controversial: although BCG provides significant protection to leprosy and can protect children from developing severe tuberculosis, its protective efficacy against tuberculosis has varied greatly in different trials (reviewed in 1). It is widely agreed that new and effective strategies for the prevention and treatment of tuberculosis are required, and that more effective mycobacterial vaccines are needed.

Adoptive cell transfer experiments in mice and studies in gene knock out animals have firmly established that protective immunity to mycobacteria is strongly dependent on MHC class II restricted CD4+ T cells and type-1 cytokines. Animals lacking functional genes encoding H2-Ab (MHC class II-/-; CD4+ T cell deficient), IFN-γ, IFN-γR, TNF-αR or IL-12p40, as well as animals treated with neutralizing TNF-α or IL-12 antibodies are much more susceptible to infections with *M.bovis* BCG and *M.tuberculosis* than control littermates. Similarly in humans, defects in CD4+ Th-1 immunity, type-1 cytokine production or type-1 cytokine receptor signaling are strongly associated with progressive infection and bacterial dissemination (submitted). An essential requirement for effective vaccines in tuberculosis, therefore, is the induction of efficient CD4+ Th-1 immunity.

Our previous work has shown that HLA-DRB1 polymorphism plays a major role in dictating the specificity and magnitude of the human CD4+ T cell response to mycobacterial antigens (2). HLA-DRB1*0301 (DR3) is a major class II allele that is present in over 20% of the human population. HLA-DR3 is associated with high responder (tuberculoid) leprosy and with strong T cell activity to mycobacterial antigens, in vitro and in vivo (2). Moreover, HLA-DR3 restricted *M.tuberculosis* reactive Th-1 cells frequently respond to the immunodominant hsp65 (3) and recognize a single dominant peptide epitope of this antigen, peptide 3-13. The hsp65 p3-13 epitope contains a DR3 allele specific binding motif and binds uniquely to HLA-DR3 but not to any other HLA class II molecule (3).

HLA class II transgenic mice as new models for vaccine design

Because HLA class II genes thus play a major role in controlling the specificity of the human T cell response to mycobacteria, the immunogenicity and protective efficacy of candidate human vaccine antigens needs to be defined in the context of HLA polymorphism. To this end, we have used recently generated HLA-DRA/DRB1*0301.Ab^0 tg mice that express HLA-DR3ab molecules. Because these mice are devoid of any mouse class II molecules expressed at the cell surface, all CD4+ T cells are restricted by the human HLA tg molecule. Immunization of HLA-DR3.Ab^0 tg mice with hsp65 protein, hsp65 p1-20 or hsp65 pcDNA plasmid, each led to efficient T cell responses to *M.tuberculosis*, comparable to BCG immunization. BCG immunized DR3.Ab^0 tg mice responded to precisely the same p1-20 epitope as human T cells. The same epitope appeared to be recognized also upon DNA immunization. In contrast, HLA-DQ8.Ab^0 mice responded to other hsp65 peptides. Peptide/HLA binding experiments revealed p1-20 as the highest affinity HLA-DR3 binder among all hsp65 peptides, suggesting a strong association between a peptide's MHC binding affinity and its immunodominance for MHC restricted T cells. In contrast, immunization with other HLA-DR3 binding hsp65 peptides (that displayed 1-10 fold lower DR3 binding affinity) induced peptide specific but not *M.tuberculosis* reactive T cell responses, thus identifying these peptides as cryptic epitopes.

Thus, HLA class II tg restricted CD4+ T cells recognize the same immunodominant antigens and processed peptide epitopes of *M.tuberculosis* as human T cells, regardless of the mode of immunization. The results thus demonstrate the major role of HLA class II molecules in dictating the antigen and epitope specificity of the T cell response and underscore the usefulness and potential of HLA class II tg mice as preclinical vaccination models.

Identification of T cell stimulating antigens with synthetic peptide libraries

The definition of novel T cell stimulating antigens of *M.tuberculosis* will be relevant to vaccine design and also for the development of new strategies for the early detection of *M. tuberculosis* infection. Different general approaches have been described by which stimulating natural antigens could be identified that are recognized by specific T cells. These methodologies can be divided in biochemical (for instance: peptide elution from HLA molecules, chromatographic or electrophoretic fractionation, etc.) and genetic approaches (for instance screening of expression libraries).

Recently, progress has been made in the use of synthetic techniques to determine the epitope specificity of T cell clones. We have described a new method for the identification of CD4+ T cell epitopes based on dedicated synthetic peptide libraries (4). Stimulating T cell epitopes defined by such synthetic libraries do not represent natural epitopes but rather are mimics thereof. This is due to the limited size and complexity of the used synthetic libraries, such that not all possible random peptides will be present in a given library. Importantly, however, these mimicry epitopes can be used for the identification of natural epitopes. The libraries we have used are designed on the basis of HLA allele specific peptide binding motifs (ref. 3) such that the library contained peptides are strongly enriched in HLA class II binding ligands. For each class II molecule, a dedicated library is synthesized. Individual 14-mer peptides are synthesized on beads in

a one-bead-one-peptide fashion. The complexity of a given library is $8*10^6$ peptides (thus containing also $8*10^6$ beads). The screening is based on three selection rounds using partial release of peptides from the beads, and subsequent sequencing of the remaining peptide attached to the bead.

HLA-DR3 dedicated synthetic peptide libraries were synthesized and screened with three DR3 restricted CD4+ T cell clones. The antigen specificity for the DR3 restricted clones had already been determined previously. In case of two T cell clones, two distinct peptide sequences could be identified in the library that were both stimulatory. Interestingly, those two sequences were very homologous to each other. In addition, these sequences shared high homology with their natural counterparts. This enables the definition of natural T cell antigens based on the identification of mimicry epitopes from dedicated synthetic peptide libraries, if the natural antigen is contained in a protein of DNA database, such as is the case for the pathogen *M.tuberculosis*. In its most simple form, this can be accomplished by a standard homology search such as in the case of a particular hsp65 peptide. However, in most cases this does not lead to identification of the natural antigen. We therefore have developed another approach, which is based on a more precise definition of the mimicry residues that are indispensable for T cell stimulation, using substitutional and omission library approaches. Based on the resulting information, a database search profile can then be designed that unambiguously leads to the identification of natural antigens. This approach was used successfully in identifying another *M.tuberculosis* (85B) antigen as well as in identifying the type-1 diabetes associated autoantigen GAD65 (N. Schloot et al, personal communication). A similar approach has been used succesfully in the context of HLA-DR1 (ref. 4).

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HOST GENETICS AND TUBERCULOSIS

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Introduction

It is now seventy years since the first evidence of human genetic susceptibility to tuberculosis was obtained in a study of twins. Progress since has been substantial but intermittent. In the last few years there has been renewed interest in identifying the genes that underlie variable genetic susceptibility to tuberculosis following the introduction of a new method of whole-genome linkage analysis. In this short review I shall summarise some of the areas of current interest in the host genetics of tuberculosis and outline some of the new opportunities. Analysis of host genetics now provides for a better understanding of pathogenesis and protection in this disease. In the context of this volume I shall highlight opportunities of particular relevance to tuberculosis research in India.

Evidence of Genetic Susceptibility

Several different types of evidence have indicated that there is an important host genetic component to variable tuberculosis susceptibility. This was first suggested by the observation that TB appears to cluster in families. However it is difficult to determine the extent to which familial clustering may be due to shared environment or shared genes. Nonetheless a better estimate of the extent of increased risk in first degree relatives compared to the general population would be of value. This measure, known as lambda-s, is now frequently used in genetic studies of complex disease as a measure of the overall host genetic component to susceptibility.

The observation that tuberculosis outbreaks may be more severe in populations previously unexposed to TB has been seen as a reflection of less genetic resistance in such "Virgin soil" populations. Studies in North America particularly by Stead and colleagues (1990) indicated that American blacks are probably more susceptible to TB than Caucasians, providing further evidence of the relevance of host genetics. More recently studies of pedigrees in which multiple individuals are affected by TB have suggested that just one or two major genes may be involved in TB susceptibility, but the assumptions and methodology used in such complex segregation analysis are debatable. Thus the best evidence that host genetics is of importance in tuberculosis comes from twin studies. Comparisons of the concordance of monozygotic and dizygotic twins is a classical method of obtaining an estimate of the genetic component of susceptibility to a complex disease and several studies were undertaken of tuberculosis in Europe and North America between the 1920s to 1950s (see for example Comstock 1978). These consistently reported a significantly increased concordance rate in identical (30-60%) compared to non-identical twins (10-20%) with about a three-fold increased risk in the former compared to the latter.

Unfortunately no large twin studies of TB have been reported from other continents and apparently none undertaken in the last forty years. It would be valuable to undertake such a twin study in Indian populations ideally in the context of a total population survey, for example using a twin registry. A study of twins with leprosy in India in the 1960s by Chakravartti and Vogel provided important evidence of the extent of genetic susceptibility to that mycobacterial disease.

It is important to note that almost all studies have been of genetic susceptibility to diseases as distinct from infection, reflecting the difficulty of reliably detecting infection and non-infection with *M. tuberculosis* in humans.

MHC associations

There have been more studies of major histocompatibility complex genes than any others in human tuberculosis. In the mid-1970 de Vries and colleagues (1976) reported evidence of non-random segregation of HLA types to affected sibling pairs in family studies in India. The HLA-DR2 type was found to be preferentially transmitted to offspring with tuberculoid leprosy. In 1983 Singh and colleagues reported that same HLA class II type was associated with pulmonary tuberculosis in family studies. These early observations have now been amply confirmed. For example Rani et al. (1993) found a strong association of HLA-DR2 subtypes with both types of leprosy and Brahmajothi et al (1991) in a large case-control study of tuberculosis in South India reported a strong HLA-DR2 association. Recently Zerca et al. (1996) have found that the HLA-DR2 association with leprosy may be related to arginine at particular positions of the HLA-DR-beta chain. Cumulatively, the evidence that HLA-DR2 alleles are associated with susceptibility to TB and leprosy in India and other parts of Asia is perhaps the most consistent and impressive in all of the literature on HLA and infectious diseases.

The challenge now is to attempt to understand the molecular basis of these diseases associations and discover what they reveal about pathogenesis of these diseases. For example, are individuals with HLA-DR2 more likely to show a TH2 rather than TH1-type immune response after infection with *M.tuberculosis or M. leprae*. Molecular and cellular immunological studies to address such issues are in progress and some of this work is reviewed by others in this symposium. The recent completion of the entire *M.tuberculosis* genome sequence provided a new approach to analysing the mechanism of the HLA-DR2 association. For the first time it is now possible to search the entire *M. tuberculosis* genome for sequences that may encode peptides that could bind to HLA-DR2. These peptides may be synthesised, tested for binding to HLA-DR2 and studied further in individual with the HLA-DR type and *M.tuberculosis* infection. Such a "reverse immunogenetic" approach has proved valuable in studying other infectious diseases such as malaria.

Other Candidate Genes :

Recently several non-HLA genes have been studied to determine whether they affect risk of developing clinical tuberculosis. In The Gambia an association between carriage of certain variants of the NRAMP1 gene and susceptibility to pulmonary tuberculosis has been found (Bellamy et al. 1998). The most significant association was with a TGTG four base pair insertion/deletion polymorphism in the 3'-untranslated region of the gene. Interestingly this gene was first identified in mice where variants affect susceptibility to Leishmania and Salmonella infections and to some strains of BCG.

In the same Gambian population variation in the vitamin D receptor was associated with susceptibility to tuberculosis (Bellamy et al. manuscript submitted). Individuals homozygous for variants at the 3`end of the gene were found less frequently amongst 400 cases of tuberculosis compared to controls. The same genotype has been associated with tuberculoid leprosy in a study in Calcutta India, in which the other homozygous genotype was associated with lepromatous leprosy (Roy et al. manuscript submitted). It is tempting to speculate that this vitamin D receptor polymorphism may affect the TH_1-TH_2- characteristics of T cell responses to mycobacteria. There has been previous clinical, epidemiological and cell biological data indicating that vitamin D may affect the course of *M. tuberculosis* infection.

Other candidate genes studied have included the tumor necrosis factor gene, variants of the mannose-binding protein gene and the interferon gamma receptor gene. However there has been no clear evidence as yet that these genes affect tuberculosis susceptibility in our studies in Africans.

Genetic Linkage Studies :

An alternative approach to identifying susceptibility genes for tuberculosis is to undertake a genome-wide search using genetic linkage analysis of families with more than one case of tuberculosis. Analysis of families with two affected siblings and unaffected parents is preferred. Highly polymorphic microsatellite markers are used to track the inheritance of chromosomal segments from one generation to the next. If one of these markers is within about 10 centimorgans (about 10 million base pairs) of a tuberculosis susceptibility gene, the affected siblings should show an increased rate of sharing of genotypes defined by that marker. Large numbers of families, usually hundreds, are required to show convincing evidence of such genetic linkage in this manner and further work is required to identify the gene after mapping its approximate location.

However this approach offers the possibility of identifying previously unknown genes that have a major effect of tuberculosis susceptibility. The approaches of analysing candidate genes and performing linkage analysis are complementary and in most multifactorial diseases it is of advantage to perform both in parallel. In Oxford we have undertaken whole genome scans on tuberculosis families from Africa (Bellamy et al. in

preparation) and, in collaboration with R Pitchappan (Madurai) and S Ghei (Agra), on leprosy families from South India. Several chromosomal regions have been identified that show evidence of linkage to these diseases and further work attempting to identify the relevant genes is in progress to identify the relevant genes is in progress.

Prospects and Opportunities : The recent increase in interest in the genetic analysis of complex disease results from major advances in human genome mapping and gene identification. This provides several opportunities for studies of the genetics of TB susceptibility. Much of this work might very productively be undertaken in India. The first evidence of association and linkage of the HLA-DR2 type to leprosy and tuberculosis susceptibility came from studies in India. The only large twin study of leprosy has also been from India. It is disappointing that no significant twin study of tuberculosis has been undertaken in the last forty years and apparently none ever in Asia. A twin registry in an Asian population with a high incidence of tuberculosis could provide valuable information on the host genetic contribution to various clinical phenotypes of tuberculosis.

This would provide useful background data for attempts to identify non-HLA genes affecting TB susceptibility in India using family studies. Recent work on leprosy in India has demonstrated the feasibility of this approach. Although work is in progress on family studies of tuberculosis in Africa it is likely that important differences in susceptibility genes will be found between Africa and India. For examples in The Gambia the HLA-DR2 type is not associated with tuberculosis (unpublished data). Also there maybe a differential association of mannose-binding protein with tuberculosis in Africa and India (see Selvaraj et al., this meeting). In another major infectious disease, *P.falciparum* malaria, it is clear that many susceptibility genes differ between African and non-African population. Recent advances in molecular immunology also provide opportunities for a detailed analysis of the possible mechanism of the HLA-DR2 association with susceptibility to tuberculosis and leprosy in India. Already studies of HLA-DR2 subtypes have been reported and studies of cytosine profiles are in progress. With efforts to identify non-HLA genes for clinical tuberculosis, studies are underway to identify non-HLA genes for clinical responses to mycobacterial antigens. These sets of genes will probably overlap to a significant degree. Once these genes are identified, comparisons of their allele frequencies between populations where BCG has shown high and low efficacy may be valuable.

Potentially the most valuable dividend from applying the new technologies of genomics to the host as well as the microbial genome in tuberculosis may come from the identification of new drug targets. There is still much to learn about the molecular mechanisms of survival of *M. tuberculosis* in the human host and how these may be targeted by specific chemotherapy and an illustration of how host genetics may help is provided by recent work on an HIV resistance gene. It has been found that individuals homozygous for a mutation in the CC-chemokine receptor 5 gene are almost completely resistant to HIV infection (Liu et al. 1996). This is because this chemokine receptor acts as a co-receptor for viral entry and a new generation of drugs is being developed that aim

to block this co-receptor. Such weak points in the defence of *M. tuberculosis* may yet be identified by host genetic studies.

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HOST GENETICS AND TUBERCULOSIS

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Experiments on animal models have supported the notions that host genetics may play an important role in determining the immune response and capacity to contain the dissemination of infection (Benacerraf 1981). Recent studies on humans also have suggested that host genetics is important in the disease pathogenesis (Davies et al 1994, cf. Hill 1996). With the elucidation of genetics and structure of MHC, TCR and peptide binding, the role of Ir genes and TCR in immune response are generally accepted to be important in an active, adaptive immunity (Koopmann et al 1997, Pieters 1997, Ward and Qadri 1997): the classical Nobel prize winning, Zinkernagel and Doherty MHC restriction phenomenon is now explained at the 'consensus motif' level (Zinkernagel & Doherty 1997). Thus the outcome of an infection may depend on many sequential hierarchical steps involved, many of which may be genetically determined by both MHC and non-MHC linked genes.

In human infectious diseases, especially chronic ones, the host defence mechanisms involved can be classified into various sequential steps, viz. i) innate / genetic immunity, ii) adaptive immune mechanisms and iii) the resultant resistance or immunopathology. The role of innate immunity in experimental tuberculosis is explained by BCG gene (Nramp1). Recently PAMs (Pathogen associated molecular patterns) and PRRs (pattern recognition receptors) , the ability of non-peptidic antigens to induce the NK and gamma delta T cells, and the induction and regulation of co-stimulatory molecules and specific cytokines have all been shown to be important (Gruenheid et al 1997, Medzhitov and Janeway 1997, Maher and Kronenberg 1997.). The innate immunity is thus thought to play a pivotal role in determining the direction of the adaptive immune response: These have been reviewed in Current Opinion in Immunology, 1997 Feb. issue (vol. 9:1). The differential susceptibility of different strains of mouse and other animals including rabbits and guinea pigs may act at macrophage level (BCG r) and may be sufficient to overcome the infection in resistant individuals (Hurtel et al 1985, McLeod et al 1995, Abel & Dessein 1997). However, the resistant allele of the Nramp1 has not afforded resistance to virulent *M.tuberculosis* (Medina and North, 1996); thus *in-vivo* bacterial pathogenesis induced by virulent pathogen in the appropriate host only may throw light on the role of these genes in disease (cf. Smith, 1996). Genetic susceptibilities to human infectious diseases have also been reviewed recently (Hill 1996). When the innate immunity could not contain the growth or spread of the bacilli, specific immune functions take over (Medzhitov and Janeway 1997). The epitopes of the pathogens to be bound will however depend on the MHC haplotype of the host, the thymic education and maturation of the lymphocytes and the resultant T cell repertoire. The epidemiology

induced skewing of the TCR repertoire in individuals of Asian origin, has also been reported (Ramakrishnan et al 1992). It is not known whether the peptide pool generated in an APC by ingesting *Mycobacterium tuberculosis* bacilli will be the same in all the infected individuals: Lysosomal enzymes and proteosomes may play a critical role here. The preponderance of a particular cytokine during innate immune response and the resultant inflammatory process may dictate the adaptive immunity to pursue towards a particular pathway and function (Medzhitov and Janeway 1997). This may add further variations and diversity in susceptibility or resistance. Innate mechanisms may be sufficient to contain a disease in majority of the individuals. When the nonspecific, innate mechanism fails, the specific, adaptive immune response, follow.

It is now appreciated that many genes and gene products in different loci, with different functions (physiological, hormonal, enzymatic, immune response etc.) may be involved in the successful outcome of resistance (immunity) or pathology. Genome scan is one methodology to identify genes with yet unknown function involved in a disease process (Cf. Davies et al 1994). Similar genome scans have been undertaken in mycobacterioses as well (A.V.S Hill, personal communication). Here it is presumptive that unless otherwise a given individual has the appropriate genes or factors at the various indicated loci, he or she may not develop the disease. Logically, it may not be essential that one should be resistant or should have developed a protective immunity for not having a disease: Conceiving that one does not have the requisite 'susceptibility' genes is easy, this may be true especially with chronic infectious diseases. At the population level, these genes may be represented in various combinations: so a gradation of disease and clinical subtypes. This may explain why majority of the contacts do not develop mycobacterioses. The question is how to identify these high risk individuals and to induce prophylactic immunity in them. It is possible that the adaptive immune response in these diseases leads to pathology.

Another dimension added to this variability in susceptibility is the ethnic difference between various populations infected: an elegant study in humans demonstrated clear interethnic differences in infection rates, febrile episodes and antibody responses to *Plasmodium* surface antigens (Modiano et al, 1996). Southern India is known for her endogamous, ethnically distinct caste groups, characterized by unique MHC haplotypes, with very high inbreeding coefficient: these caste groups have been living in sympatric isolation in the same environment and niche for millennia (Sanghvi et al 1981). They have been subjected to the same epidemic and infections through the ages. Our studies have shown that different MHC haplotypes are predominant in different caste groups and many new genes in the complement and MHC loci have been identified in our South Indian Population (Pitchappan et al 1984, Rajasekar et al 1987, Sasazuki et al 1986, Pichappan1988, Tokunaga et al 1992, Balakrishnan et al 1996, Pitchappan et al 1996, Tait et al 1996). If an Ir (immune response) and other associated genes are unique to a population and if it is associated to a disease then the association may be identified only in that particular population (caste group). Our MHC association studies in TB and Psoriasis exemplify this aspect (Brahmajothi et al 1991, Pitchappan et al 1989).

Therefore, the caste groups of South India are the ideal models to study the immunogenetic basis of tuberculosis and AIDS susceptibility. The lessons learnt in this region may be applicable to many other developing countries and so an integrated, multicentric study in South India on host genetics, molecular epidemiology and treatment responses is warranted.

We have recently studied the immune status of the sputum positive pulmonary tuberculosis patients by i) PCR-SSOP and SSP typing of HLA-DRB1, DQA1, DQB1 and DPB1 loci, ii) *in-vitro* lympho- proliferative responses to selected synthetic peptides of 38 and 16 kDa of *M.tuberculosis* origin and their correlation to MHC and iii) RT-PCR analysis of cytokine expression during the recall memory to PPD . These papers are published individually but the highlights of these studies and relevant literature are reviewed here.

MHC and tuberculosis susceptibility:

In recent times, many studies have shown association of sputum positive pulmonary tuberculosis with HLA DR2 (Bothamley et al 1989, Khomenko et al 1990, Brahmajothi et al 1991, Bothamley et al 1992, Selvaraj et al 1996). An earlier family study from North India showed a skewed transmission of the disease with DR2 (Singh et al 1983). Recently Rajalingam et al (1996) and our study (Ravikumar et al 1997) extended the DRB1*1501 association with PTB and drug resistance. In our study DQB1*0601 and DPB1*02 were positively associated with the disease (Figure. 1). A few other negative associations (preventive fractions) have also been identified (DRB1*11, DRB1*10,DPB1*04 & DPB1*08). It has been suggested that the patients and controls with DR2 are high responders (Bothamley et al 1989, Khomenko et al 1990, Brahmajothi et al 1991, Bothamley et al 1992, Pitchappan et al 1997). This is further supported by the observation that DRB1*1501 is a high PPD responder, compared with DRB1*1502 in PTB patients but a high PHA responder in controls (Figure 2). DRB1*1501 associated susceptibility has been implicated in many autoimmune disorders and diseases like allergies, multiple sclerosis, narcolepsy, idiopathic membranous nephropathy etc. in literature. (XI and XII International Histocompatibility workshops).

Our analysis of the allelic data for their co-occurence, in patients and controls revealed that while DRB1*0601 and DRB1*1501 may predispose, DPB1*04 was protective and epistatic to the predisposing alleles: i.e., in the presence of DPB1*04, the DQB1*0601 and DRB1*1501 predisposition may not lead to pathogenesis: these allelic combinations identified in patients and controls are presented in Figure 3. Many reports have suggested an association of various DQ alleles with tuberculosis and immune responses (Bothamley et al 1992, Bothamley and Schreuder 1992). The DR and DQ alleles have been suggested to be involved in the tuberculin reactivity particularly in heterozygous combinations (Selvaraj et al 1996). In our study, many other alleles, DQB1*0201, DQA1*0102, DQA1*0103 and DPB1*04 are all high responders to Con-A, peptide 38.I and/or 38.A in patients. The polymorphisms of haplotypes bearing DR2 and

the associated DQ have been of sustained interest. Literature on Indian populations have revealed many associations unique to India and their frequency differed in Leprosy and Tuberculosis patients (Singal et al 1990, Mehra et al 1991, 1996). Arginine at positions 13 or 70-71 in pocket 4 of HLA-DRB1 alleles which include DRB1*1501, DRB1*1502 and DRB1*14, has been suggested to be associated with susceptibility to tuberculoid leprosy (Zerva et al 1996). Our study in tuberculosis patients on the association of DR2 subtypes however did not reveal such results (Ravikumar et al 1997). The alleles of various MHC loci in different population thus, may play a crucial role in peptide recognition and resulting immune response. Whether the differences determine the pathological manifestation, need to be further investigated.

Figure 1 : Relative Risks of DRB1*, DQB1* and DPB1* alleles to predispose sputum positive Pulmonary Tuberculosis in a case control study from South India.
(Ref: Ravikumar et al 1997).

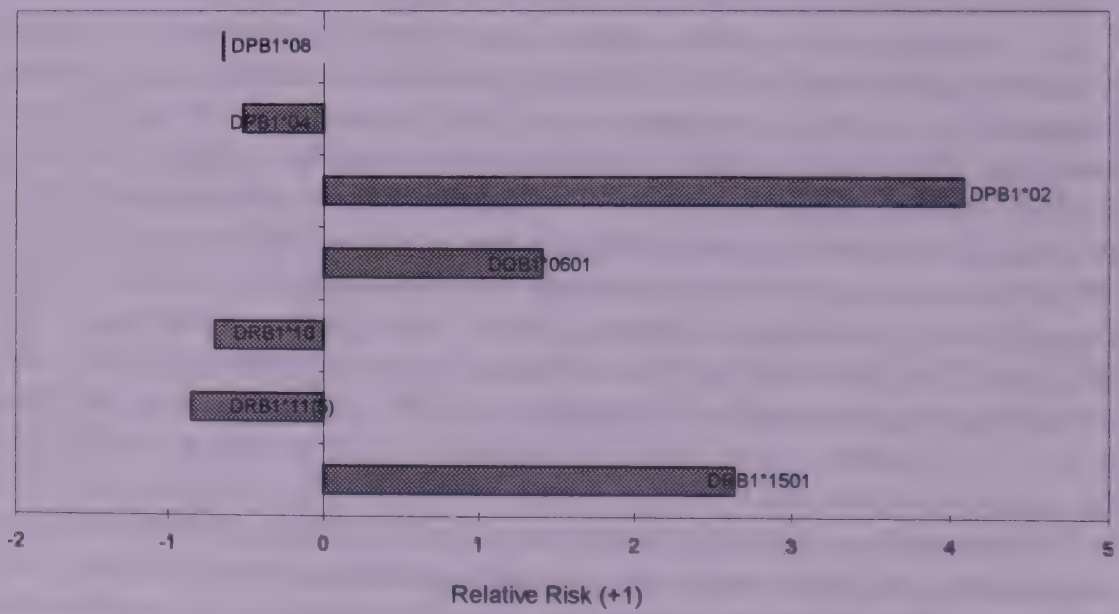
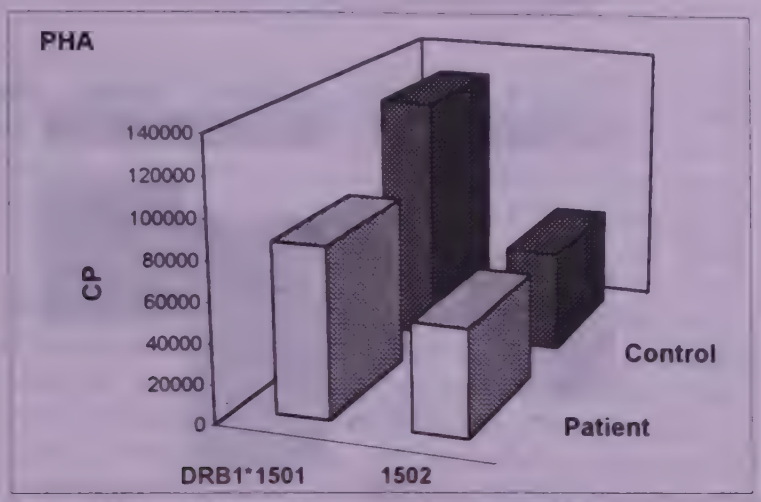


Figure 2: LTT response and DR2 subtypes in sputum positive Pulmonary Tuberculosis Patients. DRB1*1501 is a high responder compared to DRB1*1502 to PHA, Con-A and PPD in PTB, but only to PHA in controls. (ref: Dheenadhayalan et al 1997a)

PHA	1501	1502
PTB	85323	53642 t, p=0.035
CTL	125698	51201 t, p=0.015



PPD	1501	1502
PTB	32795	3899 MW, p=0.044
CTL	9137	17250 MW, p=0.352

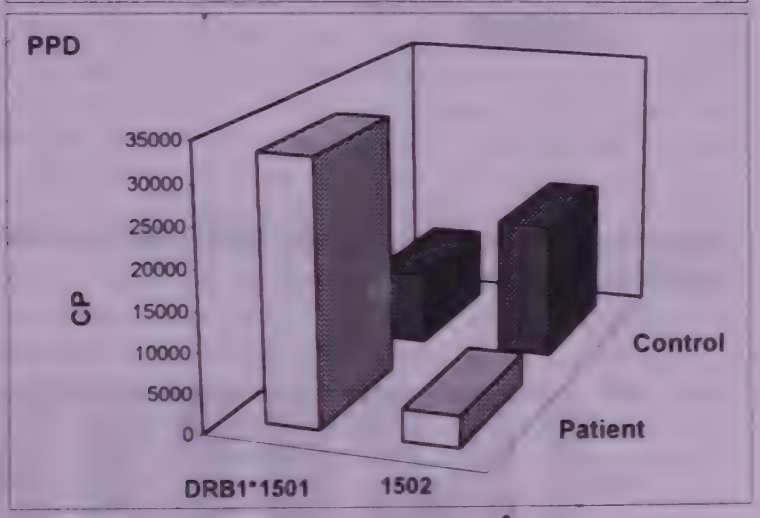
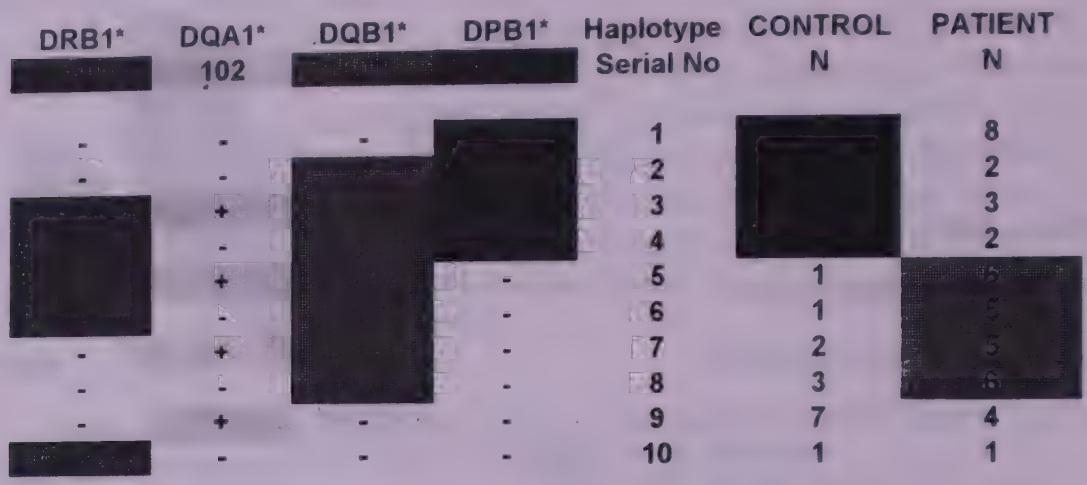
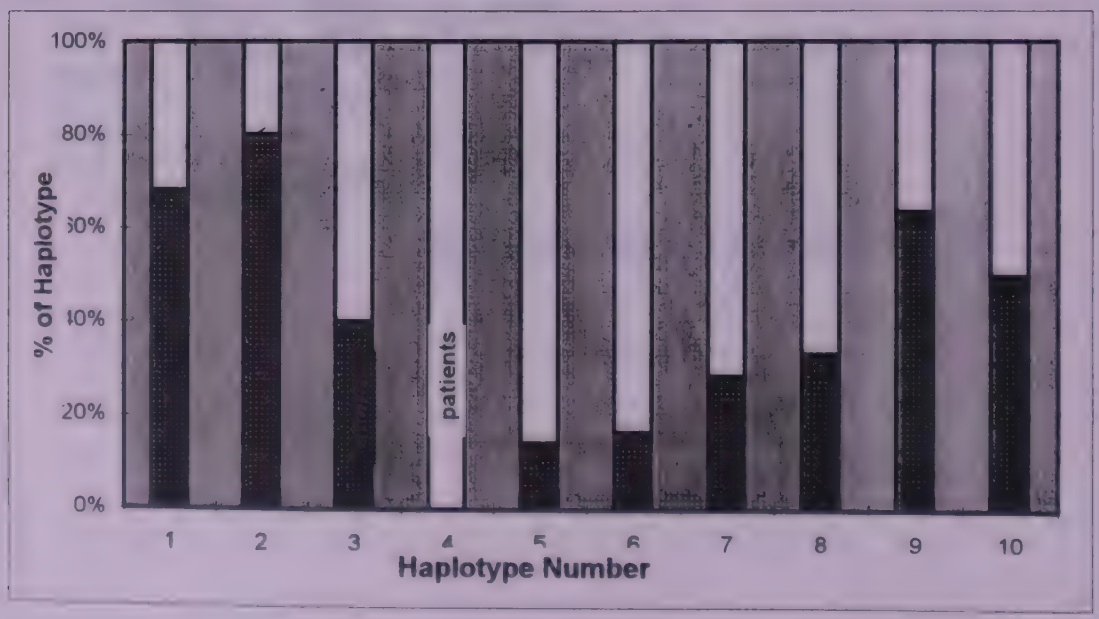


Figure 3: HLA DRB1*1501/DQB1*0601 predisposition to sputum positive PTB and the epistatic (preventive) influence by DPB1*04 (Ref: Ravikumar et al 199



Comparison of frequency of DRB1*1501-DQA1*0102-DQB1*0601-DPB1*04 haplotypes in controls and patients.



Spectrum of immune reactivity in tuberculosis and healthy individuals :

Spectrum of Immune reactivity is generally ascribed and correlated to the immune status and immunopathology in leprosy and tuberculosis: Earlier we proposed that the spectrum of immune reactivity is inherent in healthy hospital contacts (Pitchappan et al 1991): this has further been confirmed and extended in two further studies (Bothamley et al 1992, Fonseca et al 1992). These studies have now confirmed that the spectrum of immune reactivity does exist in healthy individuals from various endemic areas, viz. South India, Indonesia and Brazil (Table 1). All these studies showed an inverse correlation between Mantoux and serum antibodies: this support the general notion that the two polar forms of immune response (CMI vs HI) are exclusive of each other, though with overlaps: the contribution of this adaptive immunity in protection or disease pathogenesis is not known. It is known that the dose, route, durations, form of exposure are responsible for the preponderance of a particular type of immunity; these are nowadays explained in terms of cytokine production at the macrophage level (cf. Trinchieri 1997). Our study on tuberculosis patients and their recovery by chemotherapy revealed that the immune status of the patient decides the duration required for chemosterilization and the drug resistant status (Brahmajothi et al 1995, Lancet's Investigator Award, cf. Horton 1995, Figure 4). Early responders to chemotherapy and patients with a drug sensitive bacilli had a better CMI (Mantoux) and lower serum antibodies, whereas the late responders and drug resistants presented a relatively a low CMI and high serum antibody (Figure 4). With the recent understandings on the role of cytokines in immunity, this spectrum can be equated to the Th1/Th2 status. In Japanese children, the induration diameter of tuberculin response has shown a linear inverse relation to total serum IgE level: Further the positive tuberculin response is associated with lower levels of Th2 cytokines (IL-4, IL-10 and IL-13) and higher levels of the Th1 cytokine IFN-γ (Shirakawa et al 1996). No other confounding factor is found to influence this relationship in this study. The role of BCG vaccination, prevalence of TB and other infections, have been suggested to be responsible for this kind of correlation. It is not known whether there is any genetic preponderance of an individual to be of Th1 phenotype or Th2 phenotype to a given set of pathogens/ epitopes and whether it has any implications in disease susceptibility including tuberculosis.

Table 1
Studies on the Spectrum of Immune Reactivity in Controls and PTB Patients.

Author	Samples	Country	Skin Test	Ig isotypes	Reference
Pitchappan et al 1991	Hospital Contacts	S.India	PPD RT2	IgM, IgG	Tubercle 72: 133-139
Bothamley et al 1992	Healthy & Hospital contacts	Indonesia	PPD RT23	IgG	J.Inf.Disease 166: 182-186
Fonseca et al 1992	Healthy Subjects	Brazil	PPD RT23	IgG	Tub & Lung Dis 73:242.
Brahmajothi et al 1995	Drugresistant & recovery patients	S.India	PD RT23	IgG	Lancet Conference*
Shirakawa et al 1997	Children	Japan	Tuberculin	IgE non-specific, Cytokines	Science 275: 77-79

* Lancet's Conference on Tuberculosis, 1995, Washington D.C. Investigator award (cf. Horton 1995).

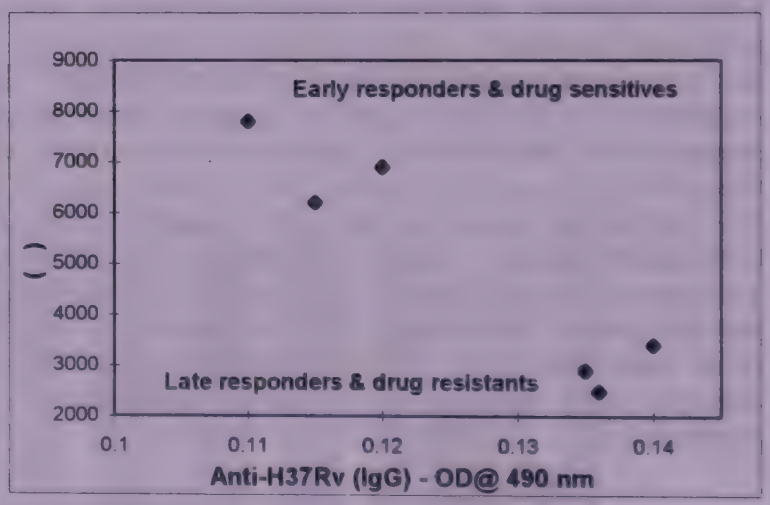
Spectrum of Immune response does exist in healthy populations from endemic area and PTB patients, i.e., DTH is inversely correlated to serum antibodies - equated to Th1/ Th2 profile (?).

This is also correlated to recovery by chemotherapy - patients with a good CMI recovering earlier and good CMI being predominant in drug sensitive patients.

The Japanese study has shown the inverse correlation between Mantoux positivity and total IgE and atopy: the cytokine levels were also compared in them: a clear Th1 and Th2 profile correlating DTH and atopy has been identified.

Figure 4: Spectrum, Drug resistance and recovery by chemotherapy
(ref: Brahmajothi et al 1995, Lancet's Investigator Award: cf: Horton 1995)

		O.D	CPM
Recovery	0-3 months	0.11	7800
	4-6 months	0.14	3400
Streptomycin	Sensitive	0.12	6900
	Resistant	0.135	2900
INH	Sensitive	0.115	6200
	Resistant	0.136	2500



Immune status of the patients at the start of chemotherpay were assessed by LTT and serum antibodies (IgG isotype) to H37Rv. Early responders to chemotherapy is compared with late respnders, Patietns with Streptomycin or INH resistant bacilli and sensitive bacilli.

BCG scar status, LTT and Th1 phenotypes:

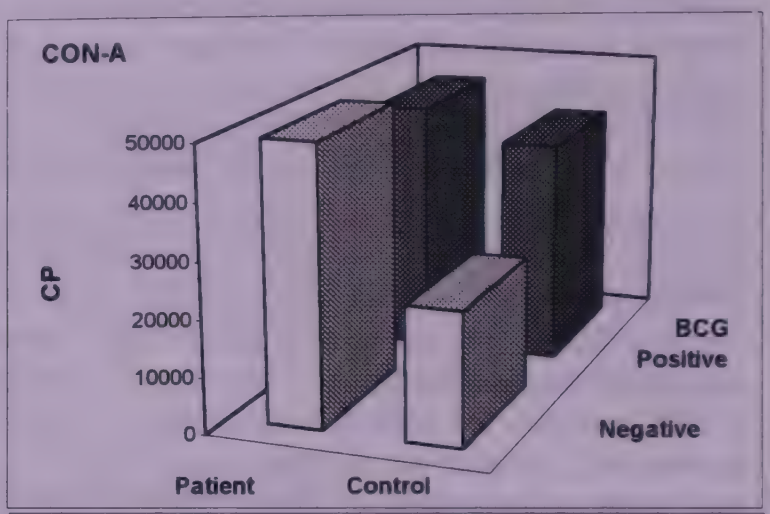
BCG vaccine is frequently accused for 'many maladies of tuberculosis' in this country: the 'culprit' may be the epidemiology, host genetics and experimental designs adopted in the investigations. This may also be due to the failure of the health care delivery system that does not transfer the fruits of the research and knowledge to common man. A vaccine for a chronic infectious disease cannot be expected to function the same way as a viral vaccine. Our earlier studies have shown that there was no correlation between the BCG vaccination status and the present Mantoux or serum antibodies in this endemic area (unpublished). Our recent studies have shown that BCG scar positive patients possess significantly elevated LTT to *M.tb*-peptide 38.1 (Dheenadhayalan et al 1997a). The Con-A response is significantly increased in BCG scar positive controls than negatives showing the influence of BCG vaccination. A similar effect of an increase in BCG positives has been observed in BCG scar positive patients than negatives, to *M.tb* peptide 38.1. (Figure 5). Cytokine expression to recall by PPD (48 hrs culture) revealed that while majority of the BCG scar positive controls are of Th1 phenotype, all the BCG scar negative control is of Th2/Th0 phenotype (Figure 6). Among the patients, both the phenotypes are represented almost equally in both BCG scar negatives and BCG scar positives. Thus, a clear polarization and a correlation to BCG vaccination has been identified in the endemic controls (Dheenadhayalan et al 1997b). The BCG vaccination has induced a Th1 phenotype in 83% of the controls studied: the phenotype in this endemic area once induced, may be persistent (mean age of the samples studied 30.3$\pm$2.4 yrs). The appearance of Th1 phenotypes in 50% of the BCG scar negative patients studied suggests the origin of this phenotype denovo: whether it is the same type of Th1 as in the case of controls is a question to ponder: It is possible that there may be two types of Th1 cells. Dissecting out these two types and the role of MHC in their development, may throw further light into the mechanism of susceptibility or resistance in this endemic environment.

Thus in this endemic area, the epidemiology (environmental mycobacteria and other infectious agents), MHC, BCG vaccination and specific infection, all seem important in deciding the course of the adaptive immune response both in controls and patients (resistants and susceptibles?). The innate mechanisms and non-MHC genes may play a role in dictating the direction of the adaptive immunity as reviewed elsewhere (Abel and Dessein 1997): but unravelling the mechanisms of adaptive immunity and immunogenetics which leads to resistance or pathology is the need of the hour. This may be important in determining the intervention strategy. The epidemiology driven skewing, the resultant MHC dependant and epidemiology dependant thymic education, the resultant TCR repertoire and usage of TCR and Ir genes in tuberculosis and AIDS susceptibility, pathogenesis and progression need to be specifically addressed. We are convinced that the South Indian caste groups with defined MHC haplotypes, with long history of living in this endemic region are the ideal models to study these immunogenetic aspects.

Figure 5: BCG scar status and LTT response of Sp+ PTB patients and endemic controls from South India. (ref: Dheenadhayalan et al 1997a)

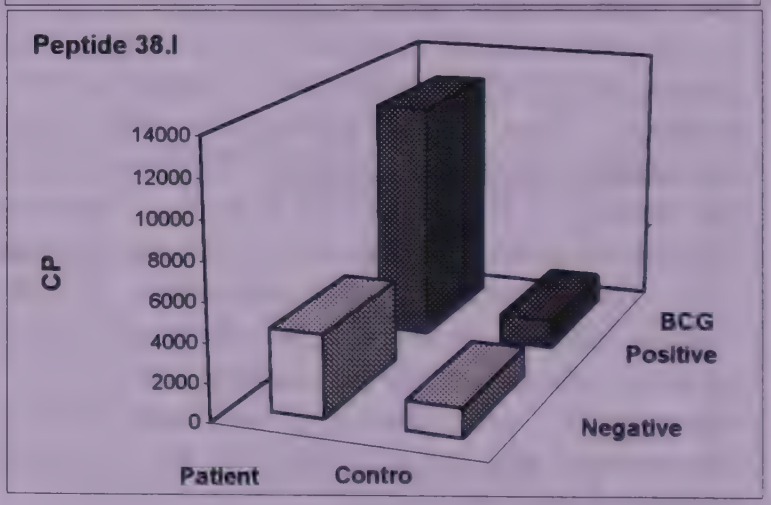
CON-A

BCG Sca	PTB	CTL	
Negative	49589	23498	t, p=0.0004
Positive	45259	39880	t, p=0.712



M.tb peptide 38.I

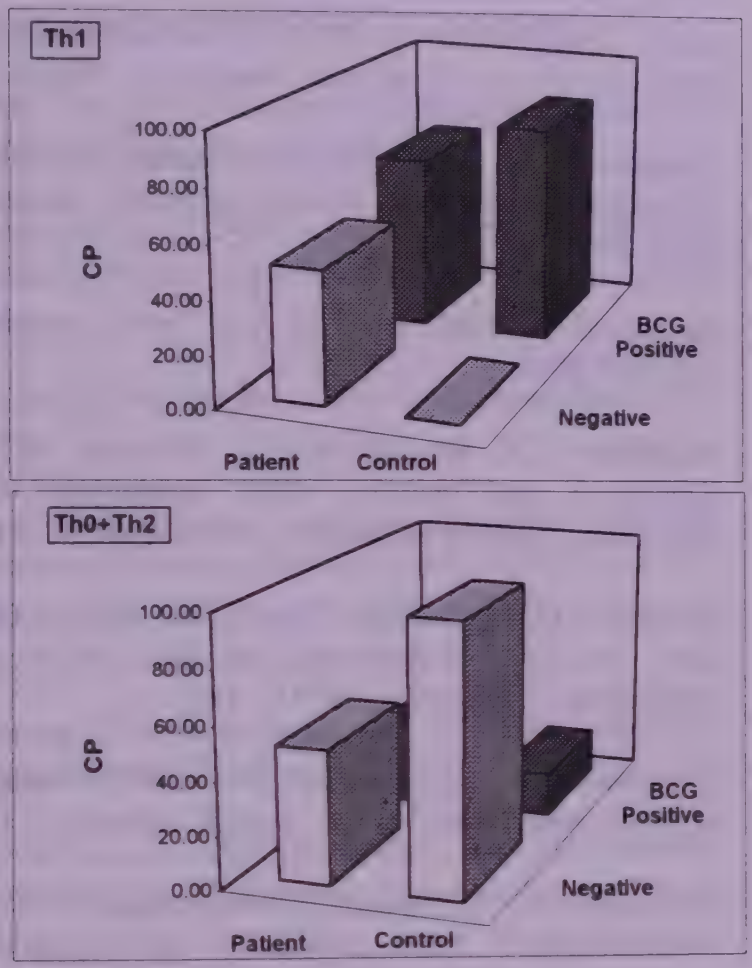
BCG Sca	PTB	CTL	
Negative	4249	1492	MW p=0.051
Positive	12457	1470	MW p=0.021



Con A response decreased in BCG unvaccinated controls:
BCG scar positive patients responded better to M.tb peptide 38.I than the other groups studied.

Figure 6: BCG vaccination status and their correlation to Th1 and Th2 phenotypes in Sp+PTB patients and endemic Controls. (ref: Dheenadhayalan et al 1997b)

		PTB	CTL
TOTAL Studied	BCG-N	18	5
	BCG-P	3	12
TH1	BCG-N	9	0
	BCG-P	2	10
%TH1	**BCG-N**	**50.00**	**0.00**
	BCG-P	**66.67**	**83.33**
TH2+TH0	BCG-N	9	5
	BCG-P	1	2
%TH2+TH0	**BCG-N**	**50.00**	**100.00**
	BCG-P	**33.33**	**16.67**



None of the BCG scar negative controls were of Th1 phenotype;
All the BCG scar negative controls were of Th2 or Th0 phenotype
Various T cells phenotypes were represented equally in patients: Disease might have skewed the phenotype.

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HLA LINKED GENES MODULATE IMMUNE RESPONSE IN MYCOBACTERIAL DISEASES

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Amongst the major infectious diseases, leprosy and tuberculosis have emerged as good examples of the influence of HLA on susceptibility to infection. The frequent finding of a deviated frequency of some HLA antigens in both these diseases suggests that such factors do play a role, probably by influencing the type of immune response that develops following mycobacterial infection. Recent studies have indicated that mycobacterial heat shock protein (HSP) antigens may represent the major targets of cell mediated immune response against mycobacteria. However, the exact HLA class II molecules required for HSP presentation to T cells are not clearly defined. The present study was undertaken to determine (i) the association of HLA-DRB1 specific amino acid sequences in leprosy and pulmonary tuberculosis (PTB), (ii) to investigate the contribution of MHC genes other than the classical HLA loci (e.g., TAP and HSP70-1 promoter region polymorphism) in influencing susceptibility to mycobacterial diseases. (iii) the HLA-DR restriction pattern of CD4+ T cell responses and cytokine release profile in response to *M.leprae* HSPs.

PCR-SSOP hybridization and sequence analysis of DRB1, DQA1 and DQB1 genes were carried out on Indian patients and healthy controls. Further, MHC restriction imposed by the phenotype of the antigen presenting cell was studied in *M. leprae* HSPs primed CD4+ T cell cultures from polar leprosy patients to identify peptides responsible for the diversity in immune reaction i.e, Th1 or Th2.

Results

The study revealed that HLA-DR2 was more strongly associated with lepromatous leprosy (LL) and drug resistant PTB, both of which are clinically severe diseases with a number of immunological similarities including production of parasite - specific antibody, depressed CMI, chronic presence of bacilli and ultimately chronic illness. More than 97% of DR2+ve patients and controls were either DRB1*1501 or *1502 suggesting that the single amino acid difference in these two alleles (Valine - Glycine) at codon 86 is not involved in influencing susceptibility to mycobacterial diseases. Sequence analysis revealed a positive association of tuberculoid leprosy (TL) with HLA-DRB1 alleles (predominantly DRB1 *15 and *1401/04) that contain Arg^{13} Arg^{70} - Arg^{71} in the DRB1 first domain. It implies that specific epitopes on HLA-DR molecules may be involved in preferential binding of pathogenic mycobacterial peptides leading to the stimulation of particular T cell clones that result in a detrimental immune response causing severe form of clinical disease. Using sequence-specific primers, significant

polymorphism could be studied in TAP1, TAP2 and HSP70-1 promoter region. Several of their alleles were found to be positively associated in leprosy and PTB. Further, *in vitro* attempts were made to define the cytokine profile of $CD4^+$ T cells from polar leprosy subjects in relation to the HLA-DR polymorphism. While the undigested HSPs and their tryptic fragments of optimal digestion could stimulate CD4+ T cells from TL patients and healthy contacts, only two fragments, 18kda of HSP65 and 3kda of HSP18 triggered $CD4^+$ T cells of anergic LL patients as well. These responses were restricted by multiple HLA class II determinants with HLA-$DRB1^*15$ providing the strongest restriction. HSP65 and HSP18 induced TH2-like activity when presented in the context of HLA-DR1 and DR7 respectively. However, when MHC phenotype of the antigen presenting cell was HLA-DR^*15 and other alleles like DR5 and DR8, TH1 like cytokine profile was obtained as determined by the release of IL-2, IL-4 and IFN-γ. Further, the 18 kda of HSP65 and 3kda of HSP18 induced only TH1 activity not only in TL but also LL patients in the context of many DR alleles, DR 15 being the main restriction element.

Conclusions

The study suggests that susceptibility to mycobacterial diseases is determined by polymorphic multiple DR alleles having common residues accommodated in 'pocket 4' of the HLA class II binding cleft. These alleles determine the functional outcome of an immune response and ultimate disease expression following mycobacterial infection. Of particular importance is the induction of Th1 like activity in LL patients in response to specific fragments of mycobacterial HSPs particularly since these patients fail to recognize *M.leprae* antigens in general.The data is relevant for planning immune intervention therapy in leprosy.

MOLECULAR BIOLOGY OF MYCOBACTERIA

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Tuberculosis is amongst the most complex bacterial disease because the pathogens grow inside host cells for long periods of time. The application of molecular biology to the understanding of mycobacterial infections began when the first reports of cloning of mycobacterial genes were published in 1985. Recently methods and techniques have been developed which enable researchers to genetically manipulate mycobacterial species. Basic research has allowed the enormous progress that has been made in molecular biology of mycobacteria in the last few years. Today mycobacterial genetics is one of the most rapidly advancing fields in bacterial genetics and this, together with the knowledge of the complete sequence of the genome of *Mycobacterium tuberculosis* open a new horizon in order to face tuberculosis with molecular biology tools.

The molecular biology of tuberculosis began by cloning and expression of genes from *M. tuberculosis* in readily grown bacterial host. First cloning host was *Escherichia coli* for which good procedures for gene cloning were available. Interesting results were obtained for the detection of probes for diagnosis where, no expression was needed, and for cloning and expression of *M. tuberculosis* and *M. leprae* genes coding for antigens recognised by monoclonal or polyclonal antibodies (Young *et al* 1985).

Cloning systems for cultivable mycobacteria to study biosynthetic pathways or pathogenesis are now available. The use of mycobacteria as hosts required the development of efficient transformation system and the construction of cloning vectors. The genus *Mycobacterium* contains fast and slow growing species sometimes very difficult to manipulate experimentally. Cloning systems for cultivable mycobacteria was developed thanks to the description of a highly competent *M. smegmatic* strain mc^2155 which has served as an ideal surrogate host for faithful expression of genes from pathogenic mycobacteria (Snapper *et al* 1990). The slow growing mycobacteria *M. bovis* BCG is used as model for *M. tuberculosis* complex.

The use of *Mycobacterium* host required the development of efficient transformation system, versatile cloning vectors including autonomously replicating plasmid and integrative vectors into the host chromosome insertion element directed or homologous recombination. Shuttle plasmid carrying selectable markers that are able to replicate in *E. coli*/mycobacteria are very useful in order to express homologous and heterologous genes, study of mycobacterial promoters activities and protein localisation. *E. coli*/ mycobacteria shuttle cosmid with cos site of bacteriophage lambda allows the cloning of larger fragments of DNA. Reporter gene systems such as the betagalactosidase, alkaline phosphatase, chloramphenicol acetyltransferase, green

fluorescent protein or luciferase allows quantification of promoters and study of gene regulation (Timm *et al* 1994). Thermosensitive conditionally replicative vectors, able to replicate at permissive temperature (30°C) but lost when temperature is shifted to 39°C are in use (Guilhot *et al* 1994). Recently the description that the counterselectable marker *sacB* is expressable in mycobacteria has improved the counterselectable vectors in mycobacteria.

Integrative vectors have been developed to promote the site-specific integration of the entire plasmid DNA into the mycobacterial chromosome. Such vectors lack mycobacterial replicon but instead carry the integrase gene and the recognised attachment site from a temperate bacteriophage allowing cointegration into the chromosome bacterial site via a recombinant event. The advantage of these plasmids is that they are efficiently maintained in mycobacteria without antibiotic selection and the disadvantage of a single copy per chromosome.

Mutagenesis systems are important genetic tools to investigate protein functions and mycobacterial virulence mechanisms. Allelic exchange by homologous recombination are rare genetic events (Colston and Davies 1994) and highly dependent on efficient delivery vectors and markers in order to circumvent relatively low transformation efficiencies in mycobacteria. New promising protocols using thermosensitive-*sacB* vectors independent of the frequency of electroporation has been recently described that could substitute the classical allelic exchange protocols by electroporating a suicide vector (Pelicic *et al* 1997).

Transposon mutagenesis generating mutant libraries of *M. tuberculosis* is today feasible. Two delivery system have been described based on conditionally replicative vectors thermosensitive /*sacB* (Pelicic *et al* 1997) and conditionally replicative phage system (Bardarov *et al* 1997). Modified transposons bearing truncated reporter genes could be used to locate promoters and study their expression. Transposition could be a powerful tool in order to identify mycobacterial virulence genes and study of the encoded virulence factors in relation to the host. The isolation of avirulent mycobacterial mutants such as auxotrophs or mutants affected in virulence genes should provide new live vaccine candidates against tuberculosis. The identification of virulence genes will also help in defining new mycobacterial drug targets.

The study of the molecular biology of the mycobacteria lead to important developments in the diagnosis and epidemiology of tuberculosis. In the future we hope it will be possible to find enzyme targets for new drug and of immunologically important molecules, the understanding of pathogenicity, and the develpment of recombinant vaccines. Genetic understanding of mycobacteria and isolation of nonpathogenic mutants will make it posibile for the construction of recombinant live vaccines.

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Mycobacterium tuberculosis : STUDIES ON GENE REGULATION, PATHOGENESIS AND DEVELOPMENT OF RECOMBINANT BCG VACCINES

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Introduction

Mycobacterium tuberculosis was discovered more than a 100 years ago, a vaccine against tuberculosis (TB) was developed about 75 years ago and chemotherapy for TB has been available for more than 3 decades. However, even at present 50,000 lives are lost due to tuberculosis every week and about one third of the world population is infected by tubercle bacilli. Emergence of AIDS and multidrug resistant tuberculosis has added further to the existing problems. The challenge for mycobacteriologists has been to apply molecular genetic approaches to understand the biology of mycobacteria and utilize this knowledge for the development of more effective prophylactic, diagnostic and curative methods to combat tuberculosis. In this paper, I describe the results of our studies on (i) the transcriptional signals and gene expression in mycobacteria, (ii) the 38K gene of *M.tuberculosis* and its association with pathogenesis and (iii) development of tools for generation of recombinant BCG vaccines.

Materials and Methods

Bacterial strains and plasmids. *M.smegmatis* LR222 (a high-frequency transforming strain), a generous gift from J.T. Crawford, Centers for Disease Control and Prevention, Atlanta, Ga., was used as the host for isolation of mycobacterial promoters. All transformations in *E.coli* were performed with strain DH5α.

Enzymes. Restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were purchased from New England Biolabs, Inc., Beverly, Mass.

Bacterial culture methods, transformation and CAT assays. *M.smegmatis* and *E.coli* were grown and transformed as described (6). *M.tuberculosis* and *M.bovis* BCG were grown in Middlebrook 7H9 broth (Difco) supplemented with albumin-dextrose complex (Difco) or on Middlebrook 7H10 agar (Difco) supplemented with oleic acid-albumin-dextrose complex (Difco) in the presence of 25 μg of cycloheximide per ml. Cells for electroporation were prepared as described earlier (9). *M.tuberculosis* and *M.bovis* BCG were transformed by using a Cell Porator (Bethesda Research Laboratories, Gaithersburg, Md.) at a field strength of 15 kV/cm. Transformants carrying the promoter clones were selected on Luria-Bertani (Difco) agar for *E.coli* and *M.smegmatis* and 7H10 agar for *M.bovis* BCG and *M.tuberculosis*, containing kanamycin (25 μg/ml) or kanamycin with

chloramphenicol (20 μg/ml). Colonies appearing on the plates containing kanamycin and chloramphenicol were used to determine CAT activity.

Results and Discussion

Transcriptional signals and gene expression in mycobacteria

A systematic approach was employed to isolate and assess mycobacterial transcriptional signals by a screening strategy that selects for promoters in a homologous environment. A promoter-probe vector for mycobacteria which utilizes promoter-less CAT gene for both detection and quantification of the strength of the promoter elements was constructed. The activation of CAT reporter gene by mycobacterial promoter-containing DNA fragments cloned upstream of it facilitates the rapid selection of promoter-containing clones by using a selection medium containing chloramphenicol (6). The results of the CAT assays indicate that stronger promoters occur less frequently in the case of *M.tuberculosis* compared with *M.smegmatis* (Fig.1). Only about 12% of *M.smegmatis* promoters could express in *E.coli*, as indicated by their ability to confer chloramphenicol resistance. All *M.smegmatis* promoters supported relatively lower CAT activities in *E.coli* than in mycobacteria. The difference varied from a marginal 2-fold to a substantial 100-fold (6). The functioning of *M.tuberculosis* promoters at a suboptimal level in *E.coli* was even more striking, as none of the promoter plasmids conferred chloramphenicol resistance on *E.coli* and exhibited little or no CAT activity in *E.coli* (6). Our data suggest that the number of mycobacterial genes whose promoter signals are recognized in *E.coli* might not be substantial (6). We have shown that fast-growing *M.smegmatis* and slowly growing *M.tuberculosis* and *M.bovis* BCG recognize mycobacterial promoters with similar efficiencies (1). This conclusion is based on the comparable CAT activities supported by several mycobacterial promoters in these species (Fig.2) (1) and is further substantiated by the observation that initiation of transcription as determined for several promoters in *M.smegmatis* and *M.tuberculosis* occurs from identical positions. These results suggest that the basic transcriptional machineries of these mycobacterial species have common determinants of transcriptional specificity (1). We, therefore, believe that *M.smegmatis* can be safely used as a surrogate host for expression of at least the constitutively expressed genes from slowly growing pathogenic mycobacteria. The situation may be different for promoters of specifically regulated genes, as observed by Timm and coworkers (22) for the *bla* gene promoter from *M.fortuitum* and the *p*AN and *psu*13 promoters from mobile genetic elements. In addition, we have carried out a comparative assessment of 'extended -10 promoters' in mycobacteria and *E.coli* which reveals that functioning of these promoters in both species is similar (2).

The alignment of mycobacterial promoter sequences upstream of the transcription start point (TSP) revealed a highly conserved, Pribnow box-like hexamer located around the -10 position in both *M.tuberculosis* and *M.smegmatis* (1). However, our results suggest that GC pressure has affected the promoter regions of *M.smegmatis* and

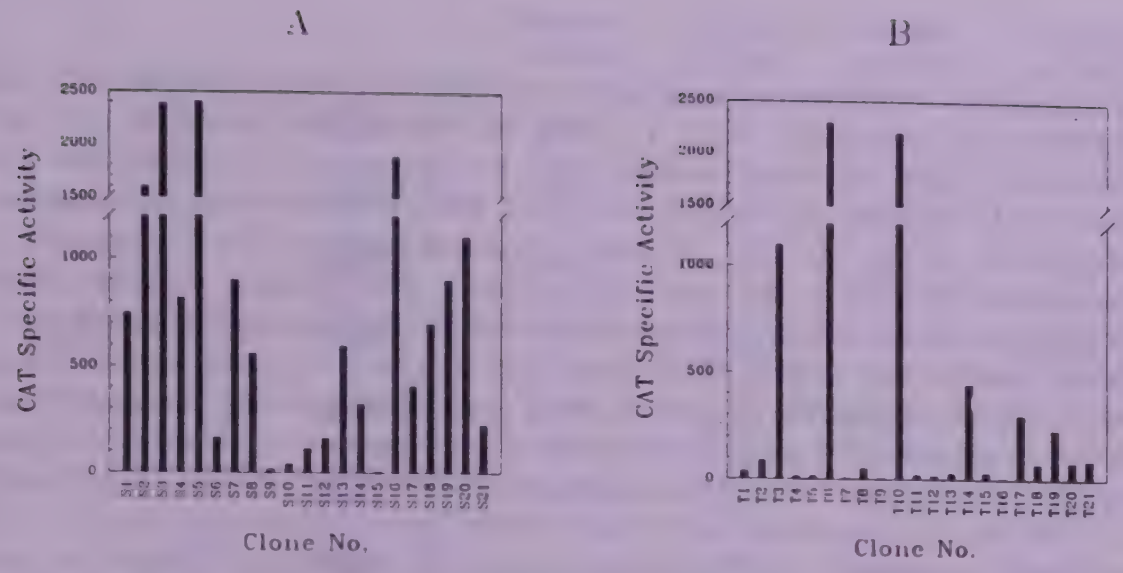


Fig.1. Comparison of strengths of *M.smegmatis* promoters (A) and *M.tuberculosis* H37Rv promoters (B) in *M.smegmatis*. The *y* axis has been broken from 1,200 to 1,500 in both panels. Promoter strength was determined by CAT assays. The CAT specific activity is expressed as nanomoles per minute per milligram of protein.

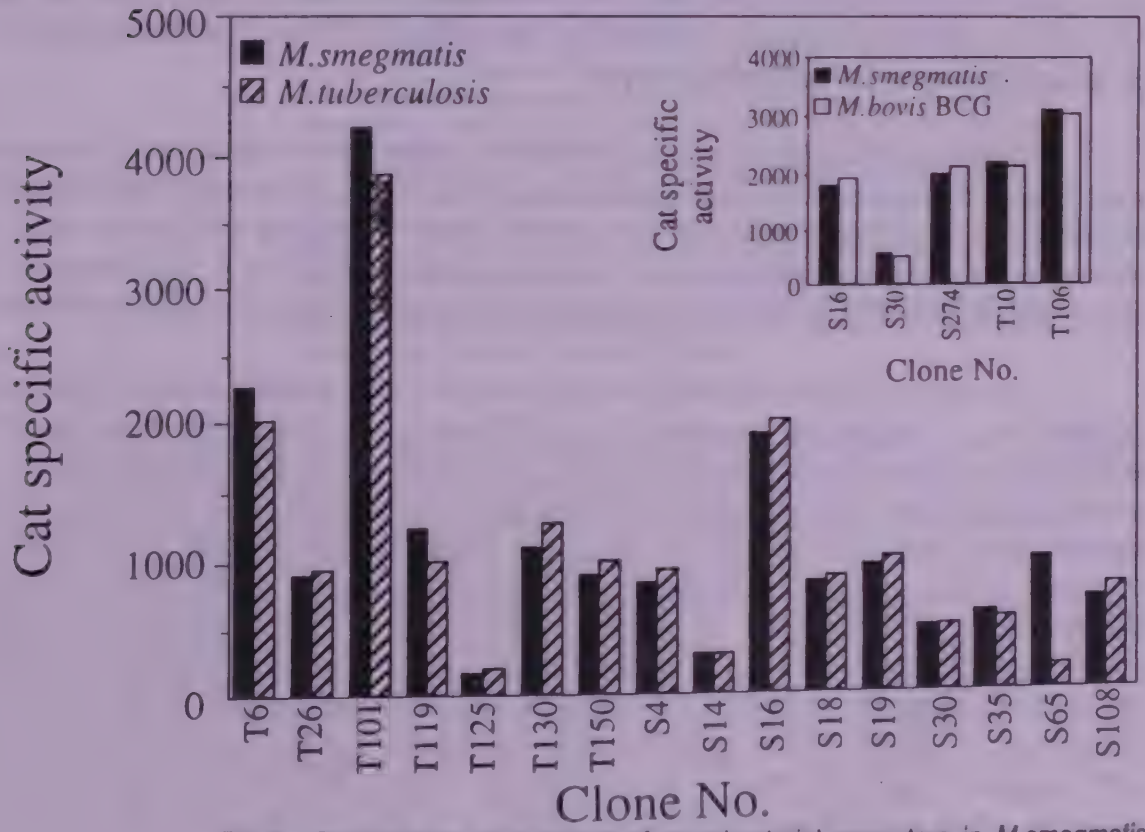
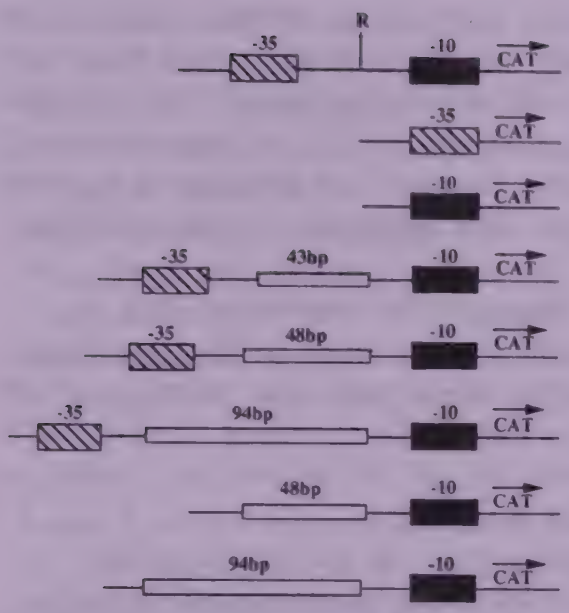


Fig.2. Comparative assessment of mycobacterial promoters in *M.smegmatis*, *M.tuberculosis* H37Ra, and *M.bovis* BCG. The CAT activity supported by *M.tuberculosis* H37Rv promoters (T6 and T150) and *M.smegmatis* promoters (S4 to S108) was determined in *M.smegmatis* LR222 (solid bars) and *M.tuberculosis* H37Ra (hatched bars). CAT specific activity is expressed as nanomoles per minute per milligram of protein. The inset shows the activities of three *M.smegmatis* promoters (S16, S30, and S274) and two *M.tuberculosis* promoters (T10 and T106) in *M.smegmatis* and *M.bovis* BCG.

M.tuberculosis to different extents, with accumulation of relatively higher GC content in *M.tuberculosis* promoters. TA---T, which like in the case of *E.coli* may represent functionally more important positions, have been conserved in both species, however, under the GC pressure in *M.tuberculosis* other bases have incorporated mutations in the direction of AT to GC (1). Sequence comparison within *E.coli* promoters reveals considerable diversity in the conserved hexamers, although these promoters must have structural features necessary for interaction with RNA polymerase in common (15). A similar situation may exist in *M.smegmatis* and *M.tuberculosis* promoters with respect to the -10 region, despite the differences in GC content between the consensus sequences. This is substantiated by our observation that a given promoter is recognized equally well by RNA polymerases from *M.smegmatis* and *M.tuberculosis* H37Ra (1). The genomic GC contents of *M.smegmatis* and *M.tuberculosis* are comparable (4); hence, a high GC content in the promoters of *M.tuberculosis* may have been selected during the course of evolution. It is not clear at present as to what is the driving force for the selection of the higher GC content in the promoter regions of *M.tuberculosis* compared with *M.smegmatis*. Additionally, *M. tuberculosis* promoters are usually weaker than *M.smegmatis* promoters (6), which could in part be the consequence of higher GC content of *M.tuberculosis* promoters. Being an intracellular parasite of humans, *M.tuberculosis* evades the immune response of the host by staying dormant in macrophages; hence, weaker promoters may be advantageous to its pathogenic nature.

Among the sequences present in the -35 regions of mycobacterial promoters, we were unable to find a single strongly conserved sequence. Although some promoters in mycobacteria have been reported to carry in the -35 region a sequence that closely resembles the typical prokaryotic -35 region consensus TTGACA, the absence of this *E.coli*-like sequence appears to be a distinctive feature of mycobacterial promoters (1).

In order to understand these observations we changed the distance between the -10 and the -35 region by inserting three different DNA fragments from the MCS of pGEM5Zf(+) with sequence lengths of 43, 48 and 94 bp in both orientations (1). The modified constructs retained variable CAT activity as shown in Fig.3 indicating that either the sequence in the inserted DNA fragment might be contributing an alternative -35 sequence or the original -35 sequence was still acting inspite of the distance created by the insertions. If the original -35 sequence was acting in spite of the insertions then one would expect comparable CAT activities for any insertion in either orientation. However, insertion of a fragment in both orientations showed drastic difference in the resulting CAT activities (1). These results again strongly suggested that the sequence in the inserted DNA fragment was contributing an alternative -35 site, thus resulting in different CAT activities when inserted in different orientations. A clear evidence of this was provided when we deleted the original -35 sequence from four of these modified constructs. It was observed that the deletion of original -35 region did not affect the activity of these constructs (Fig.3) (1). Therefore, alternative sequences generated at the -35 position rather than the original -35 sequence acting from a position further upstream were responsible for the observed transcriptional activity of all the modified promoter constructs. The absence of canonical prokaryotic promoter sequence at the -35 region of mycobacterial promoters supports our earlier observations that most mycobacterial promoters function poorly in *E.coli*.



Promoter construct	Cat specific activity	Promoter construct	Cat specific activity
pS35	640	pT3	1125
pS35.1	2	pT3.1	2
pS35.2	50	pT3.2	176
pS35.3	a) 314 b) 100	pT3.3	a) 771 b) 2000
pS35.4	a) 51 b) 235	pT3.4	a) 68 b) 700
pS35.5	a) 402 b) 75	pT3.5	a) 470 b) 1350
pS35.4'	a) 48 b) 231	pT3.4'	a) 70 b) 650
pS35.5'	a) 414 b) 67	pT3.5'	a) 500 b) 1450

Fig.3. Functional dissection of the -10 and -35 region of *M.smegmatis* promoter S35 and *M.tuberculosis* promoter T3. Promoter construct pS35 represents pSD7 containing promoter S35 of *M.smegmatis*, and pT3 represents pSD7 containing promoter T3 of *M.tuberculosis*. The letter R represents the *Xba*I restriction site, situated at the -20 position with respect to the TSP of promoter S35, and the *Kpn*I restriction site, situated at the -22 position with respect to the TSP of promoter T3. The definitions of the modified promoter constructs are as follows. The -10 and -35 regions were deleted from pS35 to yield pS35.1 and pS35.2, respectively. pS35.3a and pS35.3b contain the 43-bp *Sph*I-*Pst*I fragment, derived from the multiple cloning region of pGEM-5Zf(+), cloned into the *Xba*I site in either orientation. pS35.4a and pS35.4b contain the 48-bp *Nco*I-*Nde*I fragment cloned in either orientation. pS35.5a and pS35.5b contain the 94-bp *Apa*I-*Nsi*I fragment cloned in either orientation. Constructs pS35.4'a to pS35.5'b were obtained by deleting the original -35 region from the respective parent promoter constructs, namely, pS35.4a to pS35.5b. Identical modifications were performed with the *Kpn*I site in the pT3 constructs as described in the text. The CAT specific activity supported by each modified construct is expressed as nanomoles per minute per milligram of protein.

The tolerance of large variety of sequences in the -35 region of mycobacterial promoters distinguishes them from most other prokaryotic promoters. To our knowledge, such features in the transcriptional signals have been reported only for *Streptomyces* spp., which like mycobacteria, belong to the Actinomycete family. Strohl has studied 139 streptomycete promoters and shown that a majority (80%) of these streptomycete promoters do not function in *E.coli*, show no homology to *E.coli* promoters in the -35 region, and contain diverse sequences in the -35 region (21). The presence of multiple sigma factors and the potential for a high degree of transcriptional flexibility resulting from the capacity to synthesize different sigma factors is now firmly established in *Streptomyces* spp. (26). Mycobacteria also contain multiple sigma factors, which might explain the heterogeneity in the -35 regions of promoters. Two constitutively expressed sigma factors have already been identified from *M.smegmatis*, *M.tuberculosis*, and *M.leprae*, and the corresponding genes σA and σB have been cloned and sequenced from *M.smegmatis* (16). Predich and colleagues have proposed σA as the principal sigma factor of *M.smegmatis* (16). We compared the sequences of the 2.4 (responsible for binding to the -10 region) and 4.2 (responsible for binding to the -35 region) regions of σA and σB with the corresponding regions of the principal sigma factors of *Streptomyces aureofaciens* (HrdB) and *E.coli* (RpoD) to elucidate the nature of similarities and/or

differences between the transcriptional apparatus of these genera (1). The amino acid sequences of the 2.4 regions of σA and HrdB are identical and differ from the corresponding sequence in RpoD in only 3 of a total of 23 amino acids. Two of these are conserved changes, implying that the -10 binding regions in the sigma factors of these three bacteria are nearly identical (1). However, we observed a higher degree of variability in the 4.2 regions of these sigma factors. In a stretch of 39 amino acids, the sequences of σA and HrdB are nearly identical. However, σA differs from RpoD at 14 positions, of which 9 represent non-conserved changes (1). Our observation that a majority of mycobacterial promoters do not function in *E.coli* could be ascribed to poor interaction of the 4.2 region of the *E.coli* sigma factor with the -35 regions of mycobacterial promoters (1).

The amino acid sequences of σA and σB in the 4.2 region differ at 18 out of 39 positions and 12 of these changes are non conserved. It is conceivable that the differences in the -35 binding domains of σA and σB reflect on the diverse sequences present in the -35 regions of mycobacterial promoters. The total cellular transcription in mycobacteria may represent a division of labor involving RNA polymerase with associated sigma factors, each recognizing different or overlapping consensus sequences in the -35 regions. Although only one sigma factor with a single -35 consensus is responsible for transcription from all vegetatively expressed genes in *E.coli*, a large number of *E.coli* promoters exhibit sequences in their -35 regions which vary to different extents from the consensus element TTGACA. All of these sequences are, however, recognized by the *E.coli* RNA polymerase, albeit with differing efficiencies. It is, therefore, conceivable that if a cell expresses multiple vegetative sigma factors with different consensus sequence requirements for the -35 regions of promoters, the total number of sequences which can function at the -35 region of its promoters would be much larger than in *E.coli* and consequently the sequence requirement in the -35 regions of promoters may seem degenerate, as reported earlier for *Streptomyces* spp. and seen in our study on mycobacteria (1). *In vitro* transcription experiments with a number of mycobacterial promoters using reconstituted RNA polymerase containing either σA and σB should provide some definite answers. It is not clear how the presence of more than one sigma factor may be useful for these organisms, although a high degree of transcriptional flexibility may be helpful in responding to varying environmental conditions, as well as in supporting the complex series of interactions with the host.

Development of Gene Expression Systems for Mycobacteria

BCG, although used for vaccination of over 3 billion people, has not proven to be the most satisfactory vaccine. However, in spite of its lesser than satisfactory efficacy BCG still represents a safe and excellent immunopotentiating agent and hence it has been often proposed that BCG should be developed into a more potent vaccine by expressing candidate immunoprotective antigens in it. In order to achieve regulated expression of native or foreign genes in mycobacteria, a shuttle vector pSD5 was constructed as shown in Fig 4 (7). We reasoned that a modular vector would be necessary to create

transcriptional fusions of a wide variety of genes with a number of promoters. Accordingly, an expression cassette was designed with three different compartments namely (a) cloning sites for promoters (b) translational signals comprising of ribosome binding site and an ATG codon and (c) multiple cloning sites (MCS) for introducing genes to be expressed. The first two compartments are separated by translational termination codons to uncouple transcription from translational initiation (7).

The functionality of various modules provided in the vector to support the expression of cloned genes was tested by using the S30 promoter from *M.smegmatis* and the promoterless *lacZ* gene from *E.coli*. The transformants were blue in color and exhibited βGal activity of 1100 nmol/min/mg, thus confirming the efficient functioning of various compartments of the expression cassette in pSD5 (7).

The versatility of pSD5 to provide a range of expression was assessed by using mycobacterial promoters of various strengths to drive the expression of the *E.coli* gene encoding S-adenosylmethionine decarboxylase. The specific activity of SAM decarboxylase ranged from 157 to 18057 pmol/min/mg of protein, thus providing a range of expression levels varying by more than 100 fold. Western blot analysis of the extracts using polyclonal antibodies against SAM decarboxylase provided further evidence to substantiate this observation and demonstrated the ability of pSD5 to drive the expression of a gene at varied levels based on the mycobacterial promoter used (Fig.5) (7).

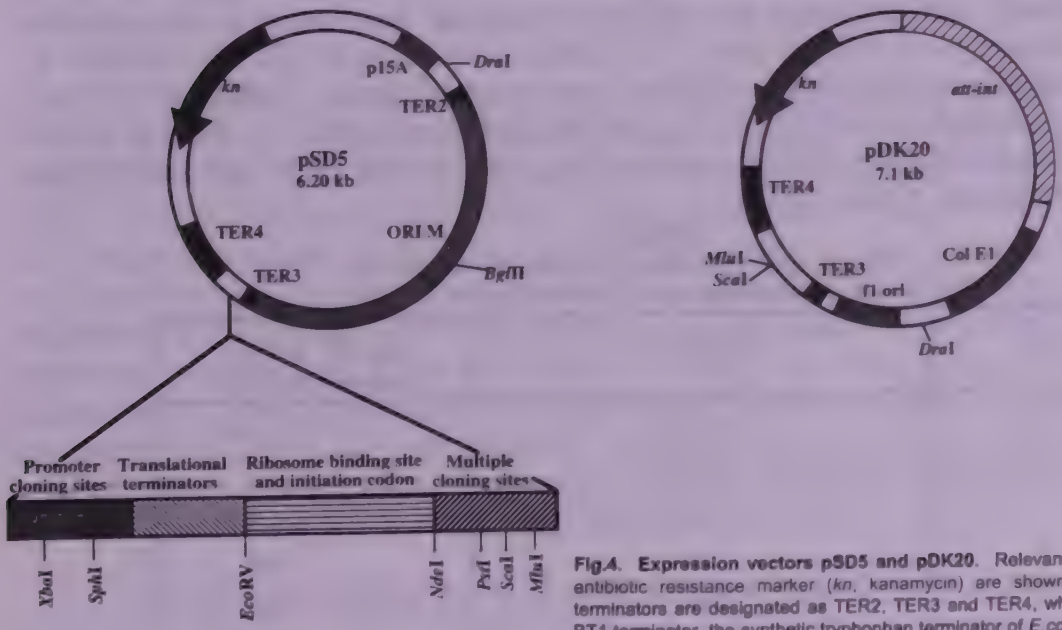


Fig.4. Expression vectors pSD5 and pDK20. Relevant restriction sites and antibiotic resistance marker (*kn*, kanamycin) are shown. The transcription terminators are designated as TER2, TER3 and TER4, which represent the rrn BT1 terminator, the synthetic tryphophan terminator of *E.coli* and *E.coli* phage fd terminator, respectively. Various modules comprising the cassette are represented by shaded bars. The unique restriction sites provided in each module are shown. The vector pDK20 carries integration sequences (*att-int*) from bacteriophage L5

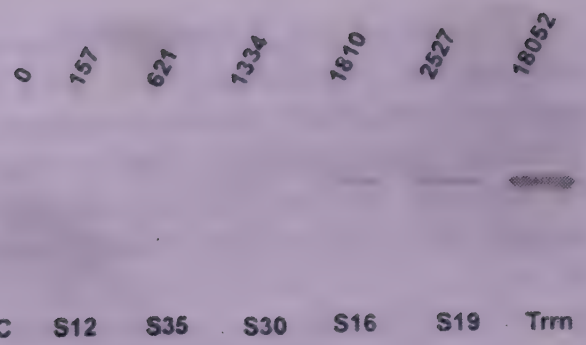


Fig.5. Immunoblot analysis of the expression of SAM decarboxylase. Cell free extracts of *M.smegmatis* LR222 transformed with pSD5.proA constructs were analysed on a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membrane and the blot was probed with antibodies to SAM decarboxylase. Lanes marked as S12, S35, S30, S16, S19 and Trm refer to extracts of mycobacterial clones carrying these promoters upstream of the gene for SAM decarboxylase in pSD5.proA. Control lane C represents the extracts of mycobacterial clone transformed with pSD5A containing promoterless SAM decarboxylase. / Numbers mentioned on the top of each lane represent the specific activity of SAM decarboxylase (pmol/min/mg) exhibited by each clone in *M.smegmatis*.

The salient feature of the pSD5 expression system lies in the versatility it provides for creating promoter-gene fusions to manipulate the level of expression as required for a particular purpose. For stable maintenance and expression of genes in *M.bovis* BCG, the nodal expression vector pSD5 was modified by replacing the origin of DNA replication for mycobacteria (ORI M) with the integration signals from mycobacteriophage L5. The resulting vector pDK20 replicates in *E.coli* extrachromosomally and integrates into the mycobacterial genome in a site specific manner to propagate as an integral part of the chromosome (Fig.4) (7). In addition, it retains the useful features of pSD5 for efficient expression of genes. Using ten different mycobacterial promoters in pDK20, βGal could be expressed in *M.bovis* BCG to levels ranging from 20 nmol/min/mg to 9000 nmol/min/mg representing a 450 fold variation in the levels of expression (Table 1) (7).

TABLE 1
Expression of lacZ gene using different mycobacterial promoters in the integration proficient vector pDK20

S.No.	Promoter	Specific Activity (nmoles/min/mg)
1	Vector	0.03
2	T23	23
3	S12	72
4	T6	294
5	T80	412
6	T3	981
7	T101	1238
8	S16	1295
9	Trm	9000

Activity of β-galactosidase supported by various mycobacterial promoters is shown. The assay for β-galactosidase was carried out as described (11).

Immunological response by the host to different antigens varies and each antigen may be required to be expressed at a particular level to elicit an optimal immune response from the host. Additionally, most immunogenic antigens of *Mycobacterium tuberculosis* are the ones which are secreted into the extracellular environment. Indeed antigens properly exported to extracellular environment *in vivo* may be presented to B-cells as well as T-cell arms of immune system in a much better way than cytosolically expressed antigens. We have used the secretory signal of alpha antigen from mycobacteria to create precise secretion translation fusions. This system enables the export of any expressed antigen to the extracellular environment. We have analysed this property by cloning the gene for β-galactosidase under several promoters in this system. The β-gal expression in the supernatants of cultures containing this secretory derivatives was detected from all promoter constructs.

These expression systems are currently being employed to express candidate immunoprotective antigens of *M.tuberculosis*, Hepatitis B and Hepatitis E virus to develop recombinant BCG vaccines against tuberculosis and hepatitis. In addition, several other derivatives of the nodal vector pSD5 have been developed to facilitate DNA manipulations in mycobacteria (11).

Studies on Pathogenesis

Pathogens usually employ several mechanisms which may act individually or in concert to produce infection and disease. Several attractive approaches are being pursued to identify such genes in *M.tuberculosis* (10). We have identified a gene from *M.tuberculosis* H37Rv that encodes a protein of 38 kDa (8).

Comparison of the deduced amino acid sequence of this gene with sequences of other proteins in the databank showed significant homology scores with VirF (12,23,24), VirFy (5,28), and FapR, Rns and Cfad proteins (3,13,18) (Fig.6A). VirFy regulates the genes required for invasion in *Yersinia* (5,28). In the case of *Shigella*, the VirF gene acts as a positive regulator of *VirB* gene (12,23,24) which in turn controls the genes for multiplication and infection of adjacent cells in addition to invasion (23). Similarly, in the case of ETEC strains of *E.coli*, adhesion and colonization is regulated by analogous regulatory proteins Rns, Cfad and FapR (3,13,18). All these proteins act as positive regulators of transcription and their binding to promoter regions is required for the transcription of genes that they regulate. A helix-turn-helix motif is present in the C-terminal region of these regulatory proteins which has been postulated as being associated with the function of these proteins (3,5,13,14,17,18,24,25).

In common with all the five regulatory proteins (3,5,13,17,18), mycobacterial protein also contains a helix-turn-helix DNA binding motif in the same location (Fig.6B). The conservation of sequence and structural similarity between these proteins is striking considering that they belong to four different bacterial genera and strongly suggests that this protein carries out similar functions in *M.tuberculosis* by regulating the structural genes involved in host invasion and establishment of the disease.

The functional implications of this gene raised the question whether this regulatory gene would be present in all species of mycobacteria. Southern blot and PCR analyses demonstrated the presence of this gene in *M.tuberculosis* H37Rv, *M.tuberculosis* H37Ra, *M.africanum* and *M.bovis* BCG, all belonging to *Mycobacterium tuberculosis* complex (Fig.7). Other mycobacterial species tested such as *M.phlei*, *M.smegmatis*, *M.avium*, *M.gordonae*, *M.intracellulare* and *M.scrofulaceum* did not possess the 38K gene sequences (Fig.7).

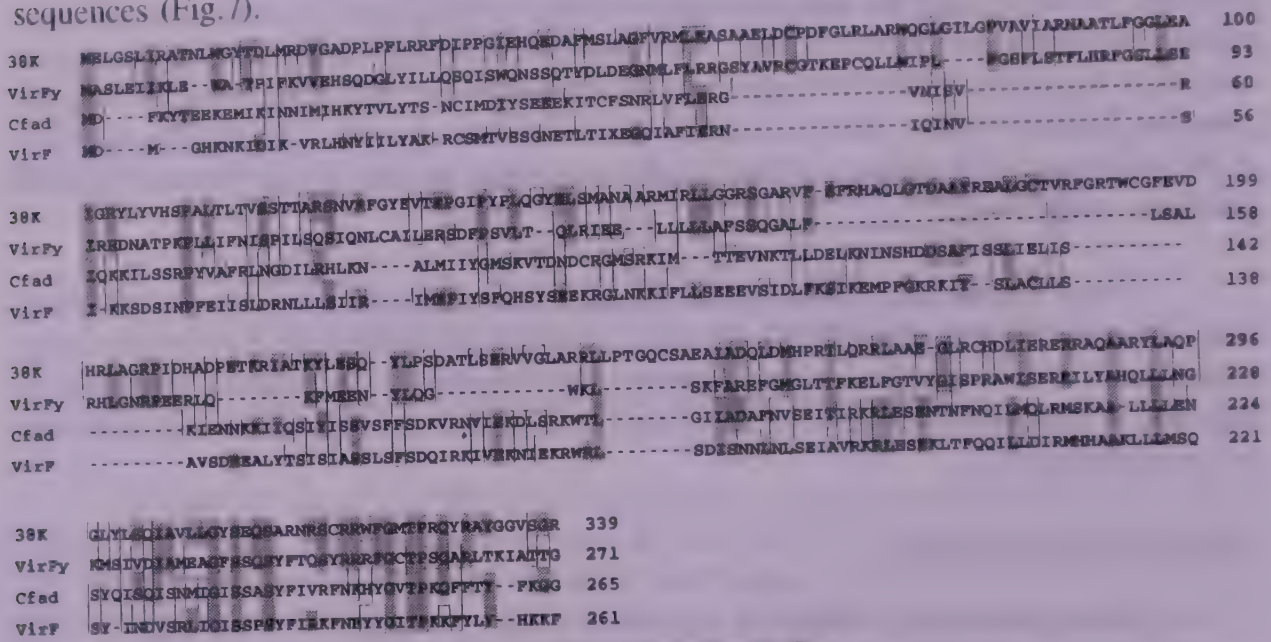
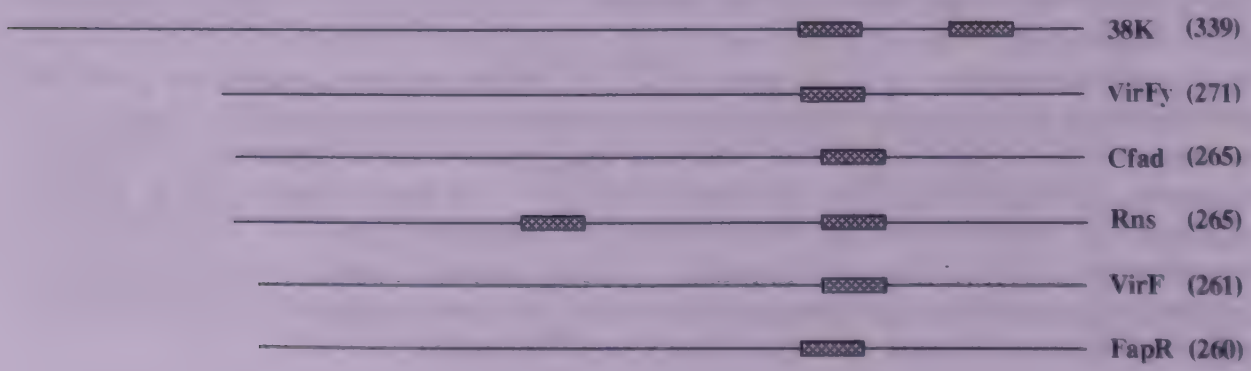


Fig.6. (A) Alignment and comparison of amino acid sequence of the 38 kDa protein with VirFy, Cfad and VirF. Amino acid sequences are numbered beginning with the amino-terminal residue of the proteins and the number at the end of each sequence refers to the total number of amino acids in that protein. Spaces represent gaps inserted to maximize matches. Residues identical to the 38 kDa protein are shaded and the functionally similar residues are boxed. Grouping of similar amino acids was as follows : HRK, GASTP, QEDN, MLVI and FYW. Alignments were made using CLUSTAL program with default parameters.

Fig 6A, TYAGI



(B) Location of helix-turn-helix motifs in the 38 kDa protein sequence. The presence of helix-turn-helix motif(s) in the sequence of the 38 kDa protein was analyzed. The plausible helix-turn-helix motifs (hatched boxes) are shown in relation to the rest of the protein (thin line). The number at the end of each line indicates the total number of amino acids in that protein.

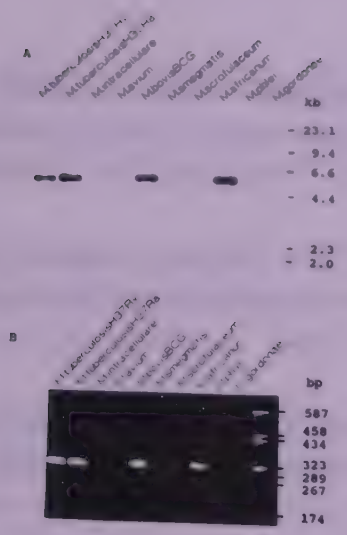


Fig.7. Prevalence of the 38K gene in different mycobacterial species. (A) Southern Blot analysis of the 38K gene. Genomic DNAs from various species of mycobacteria were digested with *Eco*RI and *Hind*III, fractionated on a 0.8% agarose gel, transferred to nitrocellulose membrane and hybridized with ^{32}P labelled fragment derived from the 3' end of the 38K gene. Hybridization was carried out at 42°C in 50% formamide. Size markers in kb are indicated on the right side of the panel. **(B) PCR analysis of the 38K gene from genomic DNAs of various species of mycobacrteria.** PCR was performed with 20 ng of DNA in a total volume of 20 µl which amplified a product of 314 bp. After completion of the reaction, 10 µl aliquots were run on a 2.0% agarose gel. The size markers (bp) are indicated.

The exclusive presence of the 38K gene in the species belonging to *M.tuberculosis* complex strongly suggests its involvement in the mechanisms employed specifically by the members of this group. In this context, it is of importance that while *M.tuberculosis* H37Rv and *M.africanum* can survive and multiply inside host macrophages, species such as *M.smegmatis* and *M.phlei* gain entry but do not grow and survive. *M.tuberculosis* reportedly uses CR3 receptors (19) or mannose receptors (20) for entry which induce the process of engulfment without activating the antimicrobial machinery of macrophage (27). On the other hand, entry through receptors such as IgF induces the host cell antimicrobial machinery and results in killing of the invading organisms (27). Considering the similarities of the 38 kDa protein with the regulatory proteins involved in invasion, survival and spread of infection, it is possible that the 38 kDa protein may control the expression of genes required by the organisms belonging to *M.tuberculosis* complex for processes that distinguish them from the other mycobacterial species incapable of entering or surviving and multiplying in the host.

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MOLECULAR BIOLOGY OF TUBERCULOSIS: A GLOBAL PERSPECTIVE

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The resurgence of tuberculosis all over the world has created interest in investigating different facets of the disease. This challenge of the disease is fortunately matched by newer technologies becoming available during the last two decades. In general, molecular techniques have provided capabilities to study in-depth the host response of diseases as well as structure and function of important genes of the pathogens. During the last 10-12 years, several investigations on various aspects of the host responses to tuberculosis as well the genomic structure and function of *M.tuberculosis* have been carried out in different countries. These studies have provided several important leads with potential for application in future. Some of the important advances in this area are discussed below.

a. Molecular biology of host immune response

The application of molecular techniques has provided significant newer information about different MHC haplotypes, target cell receptors, cytokines and lymphokines generated during infection with *M.tuberculosis*. Studies about the possible role of tumor necrosis factor (TNF) and tumor growth factor beta (TGF-β) in the tissue damage during tuberculous infection as well as also in the chronicity of the disease have yielded interesting information which may be exploited to gain further in-depth understanding of disease process as well as its management. Using these molecular methods, the immunogenic potential of several well defined antigens has also been analysed. Besides the molecules/epitopes associated with MHC haplotypes and other related genetic loci, there are interesting experimental leads about genes possibly involved in host response to infection with mycobacteria including tubercle bacillus. Among such genes of interest Bcg and Nramp I genes may provide interesting leads/directions for future research.

b. Molecular biology of *M.tuberculosis*

Due to individual effort and also because of information generated/being generated by mycobacterial genome sequencing project, our understanding of the genetic structure of *M.tuberculosis* has vastly improved. While this has provided us important information relevant to understand the physiology as well as probable mechanisms of pathogenecity of the organism, based on this information several application based techniques/strategies have already been developed.

i. Gene probes

Several DNA or RNA targeting probes for identification of tubercle bacillus have been developed from all over the world. Many of such probes have been developed into commercial systems and used by various investigators. Depending upon their sensitivity and specificity, these have been shown to be applicable at the level of laboratory identifications of bacillus as well as direct detection of sequences in the lesions. RNA targeting systems (mRNA, precursor rRNA, rRNA) have also been developed into techniques for viability testing and drug screening. Besides the systems commercially marketed, the experience with other probes (specific at genus, species and strain level) has been mostly limited to developer laboratories or some additional centres. There is clear need for investigations to know their applicability to characterise tubercle bacilli from different parts of the world.

ii. DNA fingerprinting techniques

Many RFLP analysis techniques have been developed for the characterization of *M.tuberculosis* strains. These include techniques without probes (pulse field gel electrophoresis) as well as methods based on targeting sequences like IS6110, direct repeats, spacer regions as well as methods based on rDNA region. Almost all of these systems have also been developed into PCR based techniques which are likely to be quite useful in investigating the extrapulmonary specimens directly.

Most of the experience with RFLP analysis of *M.tuberculosis* strains is with systems based on IS 6110 probes. Overall this probe(s) has been found to be quite useful and has been found to be quite discriminatory for typing of strains of *M.tuberculosis*. Using these systems interesting information about the possible evolution of strains from East Asian countries has been published.

DNA fingerprinting techniques using direct repeat probe (s) have also been found to be promising including for the characterization of strains which have limited number of copies of insertion sequences and were not typable with IS 6110 probe.

Spoligotyping using probes targeting spacer sequences has emerged as another important approach and appears to be quite promising for characterizing *M.tuberculosis* strains.

Ribotyping employing conventional rRNA probes/cloned fragments as well as PCR based approaches has been established to be quite useful in characterizing different mycobacterial strains. Our experience of developing and using these techniques shows clear potential in typing of strains of *M.tuberculosis*

It is expected that combined application of various DNA fingerprinting systems will be complementary in molecular epidemiological investigations. There is thus need to plan and carry out more of such studies in different geographical areas of the world.

iii. Gene amplification techniques

These techniques have been developed for amplification of DNA and RNA of *M.tuberculosis* for the purpose of detection and also for DNA fingerprinting. So far more than 15 gene amplification systems targeting various gene regions of *M.tuberculosis* such as IS 6110, ribosomal DNA (promoter, 16S, 23S and spacer) as well as genes encoding for various protein antigens (65 kD, MPB 64, 32, 38 kD etc.) have been described.

Most of the PCR assays are based on primers targeting different stretches of IS 6110 sequences. In some assays GC rich repetitive sequences (PGRS or GCRS) have also been targeted. Use of two repetitive elements together (IS 6110 and PGRS) as targets has been observed to be another good approach. Assay systems such as targeting mtp 40 region (which amplifies only from *M.tuberculosis* and not *M.bovis*) also could be important for specific needs. Various amplification methods developed for tuberculosis include different types of PCR (conventional DNA-targeting, RT-PCR, nested/heminested etc.), as well as assays like isothermal amplification approaches based on QB replicase and strand displacement amplification techniques. Gene amplification assays targeting ribosomal gene region have also been described to be useful. A commercially marketed system targeting this region (Roche) has been described to be quite promising.

Several gene amplification assays targeting rRNA gene region have been reported to be useful for detection, viability determination and also DNA fingerprinting.

The systems specially using the isothermal amplification strategy appear to be a good alternate route for gene amplification. The assay systems such as Mycobacterial Tuberculosis Direct Test as well the system using QB replicase enzymes need to be given attention. Similarly other isothermal amplification approaches such as strand displacement amplification appear to hold promise for future.

Emerging experience from all over the world shows special application potential for various gene amplification methods in the diagnosis of extrapulmonary tuberculosis and also in investigating the molecular epidemiology of the disease. A sensitivity of 1-10 organisms can be reached in most of these assays. Depending upon the need one can select/develop a genus specific, species or strain specific assay - the product can be further analysed by probes, RFLP or sequencing. All such approaches have been used by various investigators. There could be added advantage of identifying drug resistant mutants simultaneously.

There have been problems of contamination, cross-reactivity, lack of applicability to all strains, appropriate protocols of sampling and inhibitors. Phenol extraction of DNA from samples, use of sequence capture resins, immunomagnetic beads to concentrate the organisms are some of the approaches tried to get over the problems of inhibitors and limited number of organisms and are recommended to improve the sensitivity specially in the extrapulmonary specimens. Finally there could be the limitation of feasibility of field application. These are all methodological and logistic issues and as such do not dilute the importance of technology. All these issues can be addressed in the future research programmes by carrying out indepth preferably multicentric- developmental cum evaluation studies.

iv. Drug resistance

While assays based on molecular biological techniques have been major developed for rapid drug screening of tubercle bacillus, the major application of these techniques has been in investigating the genetic basis of drug resistance. Mutations in the target sequences associated with drug susceptibility to rifampicin, INH, quinolones, streptomycin, clarithromycin etc. have been identified by various molecular methods such as PCR-Probe hybridization, PCR-SSCP and direct sequencing of PCR products.

Mutations in genes such as rpoB for rifampicin, katG, inhA and ahpC for INH; gyrA & gyr B for quinolones; 16S rRNA and rspL for streptomycin; 23 S rRNA for clarithromycin resistance have been identified. A commercial Inno-LIPA system for detection of rifampicin resistance is available and experience with its application is accumulating. The identification of several unique pencillin binding proteins in susceptible (along with beta lactamase inhibitors) strains; enzymes and their genes for ethambutol susceptibility/resistance (emb AB gene etc.) and cluster of 4 genes encoding methyl transferases required for mycolic acid biosynthesis are new important developments in this area.

However, most of published studies show that our knowledge of molecular basis of mechanism(s) action of common drugs like INH, rifampicin and quinolones is incomplete. It is possible that other unidentified mutations, permeability barriers, drugs inactivation mechanisms could be responsible for such resistance in some strains of *M.tuberculosis*. Further role of factors such as mobile genetic elements in transfer of drug resistance (if at all this occurs) remains to be probed adequately. Emerging experience shows many gap areas for future research.

v. Antigens/molecules involved in virulence/protection

Over the years, several antigens of *M.tuberculosis* have been identified which have been shown immunogenic potential of stimulating both humoral and cellular immune responses. Genes of many of such antigens (6, 10-12, 19-22, 29, 38, 65 kD antigens etc.) have also been identified and expressed in artificial host. In addition some molecules

differentially expressed by virulent strains of *M.tuberculosis* & *M.bovis* have been described. Differences in the expression of antigens by mycobacteria residing in different host cells have been reported. Identification of siderophore binding protein, urease gene and purine biosynthesis genes are also important developments. The role of these molecules in understanding the immune response as well as to design immunodiagnostic and immunoprophylactic reagents needs to be investigated.

Dormancy or persistence of drug sensitive organisms (despite adequate dosage of drugs) is another important problem in tuberculosis and other mycobacterial infections. Molecular techniques can be used to understand the basis of such dormancy. Antigens or enzymes differentially expressed by such organisms may help in devising strategies to identify such stage and also possibly prevent and manage it. Such knowledge will be important to reduce the risk of relapses.

vi Recombinant multivalent vaccine

In the initial years, lot of interest in developing a multivalent vaccine with BCG as a vehicle was shown by various investigators. Though significant progress towards developing techniques and tools such as vectors/expression systems has been made, it may take sometime before something concrete emerges. Our understanding of relevant antigens appears to be still inadequate and further indepth studies on this aspect need to be carried out.

It is heartening that progress made in this area has laid a strong foundation towards building up a comprehensive picture of molecular biology of tuberculosis. While the knowledge accumulated from various studies and mycobacterial genome sequencing project provides a baseline information, investigations on natural wild isolates will be necessary to understand the diversity. Some of the important areas for future research could be :-

a. Application studies on gene probes and gene amplification methods on strains/specimens from different clinical forms of tuberculosis from different geographical regions.

b. Molecular mechanisms of drug resistance in the identified gap areas.

c. Virulence factors/relevant antigens relevant in the pathogenesis/protection.

d. Host & bacterial mechanisms associated with persistence/dormancy.

THE MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS

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DNA polymorphism in *M.tuberculosis* :

M.tuberculosis complex bacteria constitute a remarkably homogenous group as revealed by the inability to differentiate individual strains by multilocus enzymes electrophoresis, the minimal DNA polymorphism in restriction fragments of randomly chosen chromosomal DNA fragments and the extremely little variation in the sequences of house keeping genes. Latter observation has led to speculation that the population of *M.tuberculosis* presently found among man disseminated from a single lineage (through a *genetic bottleneck)* not more than 15,000 to 20,000 years ago. Nevertheless, the discovery of a variety of repetitive DNA elements in *M.tuberculosis* complex has led to powerful methods to differentiate clinical isolates.The most widely used element for strain differentiation of *M.tuberculosis* is IS6110, an insertion element of 1.3kb, which has the ability to move within the genome. Other DNA elements frequently used for strain differentiation are short repetitive elements such the polymorphic GC-rich repeat(PGRS, 9 bp) and the direct repeat (DR,36bp). The polymorphism associated with latter element is particularly efficient target for PCR amplification and therefore diagnosis and strain typing have been combined in a single test known as *spoligotyping*. Latter method is useful when rapid results are needed about suspected transmission in institutions such as hospitals.

Most studies have used the IS6110 element for strain typing. Usually strains were differentiated by Southern blotting of genomic DNA and using IS 6110 DNA as a probe. Although these hybridisation patterns (DNA fingerprints) from clinical isolates differ greatly in the number and the size of IS6110-containing restriction fragments, the patterns are remarkably stable when strains are in vitro subcultured for many months. Also no differences in IS6110 fingerprints have been found after long time propagation in animals. The pace of the molecular clock of IS6110 and the other elements contributing to DNA polymorphism is largely unknown. However recent data from the analysis of serial isolates from patients with chronic tuberculosis suggest that the halftime of IS6110 fingerprint may be in the order of 5 years. One of the goals of a recently initiated European Union-supported project on the molecular epidemiology and control of tuberculosis is to collect large numbers of such serial isolates and also isolates from well-documented outbreaks to estimate the stability of DNA types of *M.tuberculosis*.

Molecular epidemiology of tuberculosis : Ad hoc epidemiology

The degree of polymorphism in a population of *M.tuberculosis* is crucial to interpret DNA fingerprinting data. Clonality is most convincingly demonstrated when there is considerable background diversity in a population such that finding isolates with identical patterns implies an epidemiologic connection. Most molecular epidemiological investigations in TB have been *adhoc* studies in which cases of suspected transmission were confirmed by the indentity of DNA types. These studies have been used for outbreak investigations, to identify transmission between neighbours, in congregate living facilities, prisons, outpatient clinics and hospitals. The strong evidence of nosocomial infections obtained by DNA typing often has led to the implementation of strict control measures in case of outbreaks of TB and multidrug-resistant (MDR) TB, such as isolation, air treatment and improved docontamination of bronchoscopes. Furthermore the ad hoc approach is useful to distinguish relapse from exogenous reinfection, which may be important to assess the efficacy of drug treatment.

Population based molecular epidemiological studies

In this approach the isolates of all patients within a group (a city, province or country) are typed during a long time (2 or more years). The power of such studies is the disclosure of unsuspected transmission. In the first study of this type Small and coworkers found that less than 100% of the cases linked by identical DNA fingerprints were in fact identified by traditional methods of contact tracing. Furthermore the degree of ongoing transmission was higher than previously thought in low incidence areas. The population-based studies disclosed large chains of transmission among groups of people who have a common social behaviour such as homeless, drug users, homosexuals and HIV infected and it also showed the spill over to non-risk groups in the population. The prompt recognition by typing should facilitate arresting such microepidemics.

When strains exhibit identical DNA types these cases are designated as being clustered. Assuming that the degree of clustering of TB cases is a measure of ongoing transmission within the time window investigated, the risk factors of transmission can be investigated by comparing the clinical, and socio-demographic variables of clustered and non-clustered patients. Studies in the US identified HIV infection, socio-economic status, being born in the US, and drug resistance as risk factors of recently acquired infection. A four year study in The Netherlands among over 4000 patients showed that isoniazid-resistant strains are less transmitted in the population compared to drug-sensitive or mono streptomycin resistant strains. The calculated percentage of recently transmitted cases of isoniazid-resistant TB cases was only 19% versus 45% for drug-sensitive cases. Isoniazid plus streptomycin resistant strains were also less transmitted. In addition, the size of the transmission chains (the size of the clusters) was considerably less of isoniazid-resistant cases. These data suggest that the isoniazid-resistant *M.tuberculosis* are less transmitted, perhaps by decreased virulence due to impairment of the catalase gene.

Analysis of population based typing data allow to estimate the average number of secondary infections per source case, the *transmission index*. The parameter for the whole patient population in The Netherlands was 0.4, however, it differed significantly among different population groups. The effective reproductive rate of tuberculosis was estimated and this value was found to be below unity for the population groups, indicating that the epidemic in the Netherlands will decrease in the as long as the TB control programme remains functioning at the present efficacy.

Borgdorff and Nagelkerke at the RIVM developed a method to estimate the transmission between subgroups in population, using population-based fingerprint data. The analysis of the data in The Netherlands indicate that about half of the recently acquired infections in the general populations are from patients originating from high incidence countries. This corresponds to about 20% of all cases in the general population. The method may be used to disclose high risk groups for TB transmission and perhaps to establish the efficacy of the TB control programme for these risk groups.

By systematic fingerprinting the errors due to laboratory contaminations can be disclosed. In the Netherlands these errors contributed to about 5% over diagnosis in the laboratory, which is about equal to the level found in other laboratories. In the framework of the EU project on the molecular epidemiology and control of TB the magnitude of this problem in various European countries is presently addressed

Global spread of TB

The exquisite impact that social disruption has on tuberculosis is demonstrated by the observation that tuberculosis death rates in Western Europe doubled during the 4 years of World War I. In addition, increased international travel favours the efficient transmissions of pathogens across political boundaries. Thus, the persistence of tuberculosis and the emergence of drug resistance strains in any geographic region constitutes a global threat. It is hoped that a molecular epidemiologic approach will provide an understanding of the details of the global spread of tuberculosis and will suggest effective methods to curtail such transmission in the face of the inevitable social disruption which faces the world of today. Fingerprinting of strains from different countries has shown an association between DNA type and geographic origin. A concerted action within the European Community has recently been initiated to collect the fingerprints from many different countries and to store these in a computer database. This information may provide clues about issues such as the impact of HIV, migration, drug use and resistance or BCG vaccination on the transmission within and across country borders.

GLOBAL EPIDEMIOLOGY OF TUBERCULOSIS (WITH PARTICULAR REFERENCE TO INDIAN SITUATION)

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I. Infection & its progress in susceptibles : Experience from animal models

The epidemiology of tuberculosis in a community is the resultant of the interplay between the socio-economic state of the population, the host factor and the agent characteristics. In a manner these seem to determine the course of the events following infection in a susceptible host. It is pertinent here to recall these events, as observed in a susceptible animal host, before studying the course in human beings, followed by that in the community at large.

The over-riding tendency following the challenge with an infection by *M.tuberculosis* in a susceptible animal host, is for the bacilli to grow unhindered and exponentially at the site of entry (i.e. the lung), till say, 3 weeks or so, following a lag period of about 3 days. (Fig.1). (1) The growth then ceases and this event is nearly synchronous with the development of tuberculin positivity in the animal. Simultaneously with this, the bacilli could be recovered from the extra-pulmonary sites as well, e.g. the spleen, as also from other lobes of the lung (phase of bacillaemia). During the phase of bacillaemia in the immediate post-primary phase, bacillary implants could be lodged in the Apical and Sub-Apical regions of the lungs (ASA – regions). It is presumed that the dissemination takes place approximately at the time cell mediated immunity (C.M.I.) supervenes and bacteriostasis has already set in at the site of entry (primary site), leading to stoppage of bacillary growth at that site. Bacilli however still multiply in the primary - free lobe of the lungs, or at other sites, even as their growth stops at the primary site (Fig. 2). This is explained by the possibility of macrophage-activation locally, rather than systemically. Approximately, a period of 70 days or so is needed for the bacilli to get reduced in significant numbers at the primary site, followed by similar phenomenon at the other sites. Through CMI-activation, the site of entry is sterilised. However, the bacilli are still capable of surviving in low numbers at the ASA-regions.

Thus , it could be observed during the course of these events that the host-factor has a decisive role in limiting the effect of infection, on its own. It is postulated that subsequent development of the pulmonary tuberculosis (post-primary tuberculosis) could be the result of suppression of the CMI and consequent reactivation of the dormant bacilli harboured at the ASA – regions (Endogenous Pathway). Alternatively, it could also be conceived to be due to infection by the bacilli afresh, and in an over-whelming dose, enough to over-ride the CMI-barrier (Exogenous Pathway). The latter is considered to be a strong possibility, especially in areas with high risk of infection transmission. However, what strikes one as remarkable, in the entire process of primary and post-primary phases of host-agent interaction, appears to be the **dominant host reaction in the large majority of cases, so as to prevent tuberculosis infection manifesting as a clinical form of tuberculosis disease in the susceptible host.**

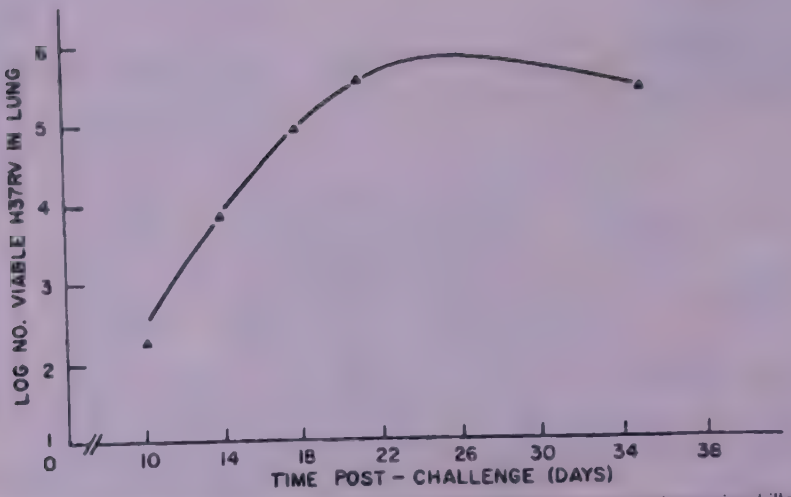


Figure 1: Number of M. tuberculosis recovered from the lungs in guinea pigs killed between 3 and 35 days after infection via the respiratory route.
Source : Smith DW. [1]

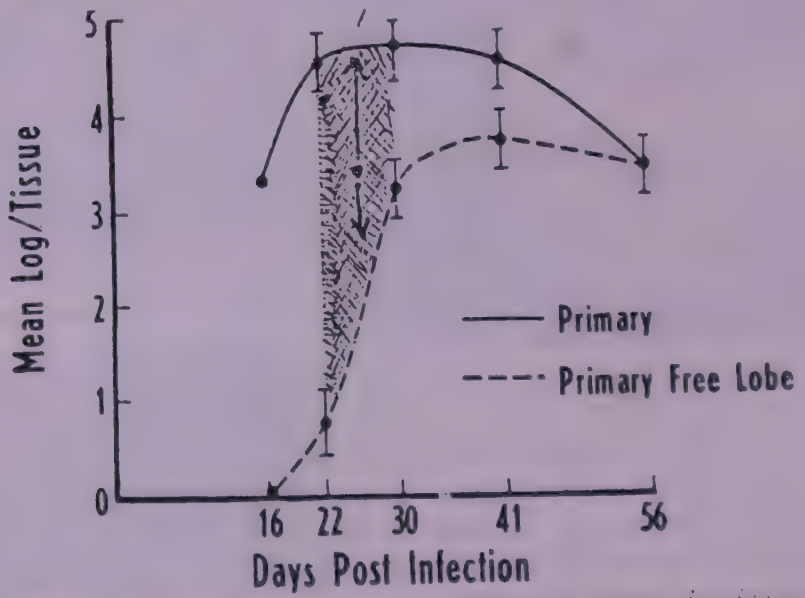


Figure 2 : Number of M. tubeculosis recovered from excised primary lung lesions and from lung lobes without primary lesions in guinea pigs killed 16, 22, 30, 41 or 56 days after challenge via respiratory route.
Source : Smtih DW. [1]

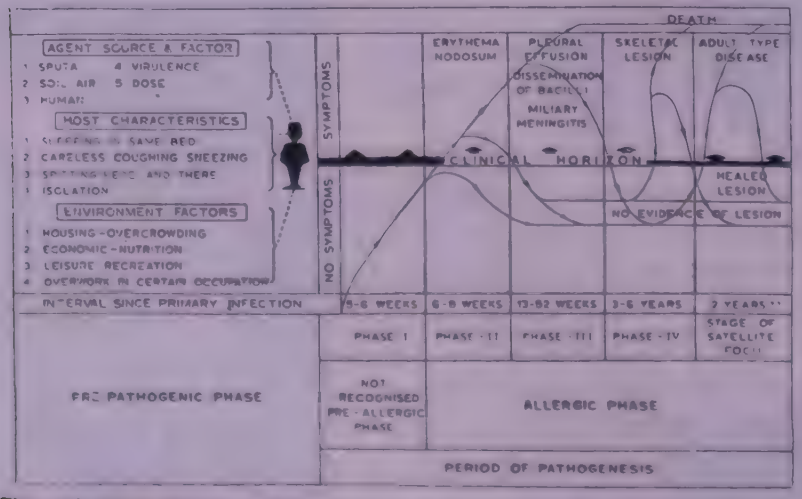


Figure 3 : Natural History of Tuberculosis (in individuals)
Source Gothi GD. [2]

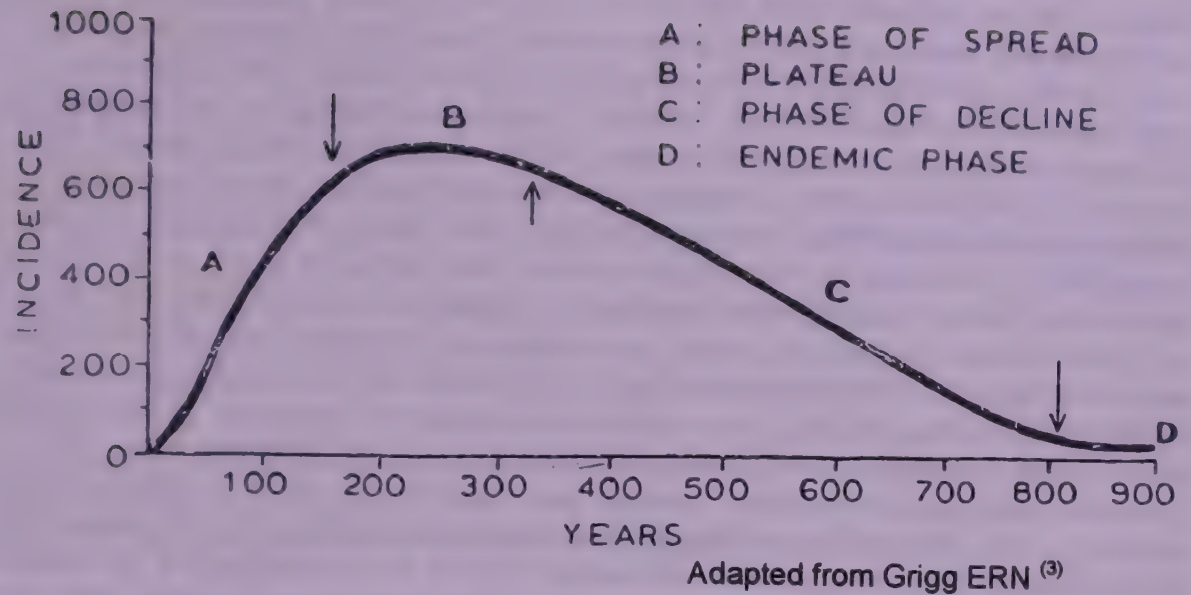


Figure 4 : Secular Curve of Epidemics
Source : Gothi GD. (2)

II :Progress of infection into clinical tuberculosis

Fig. 3 shows the course of development of clinical tuberculosis in an infected human being, during the entire period following infection.(2) Primary infection is mostly a silent event and so could be many of the events taking place in the immediate post-primary phase. These go unrecognized. It is only when symptoms develop, that a person is subjected to diagnostic tests and is amenable to diagnosis. Not only that, most of the clinical forms tend to be cured in a natural course of events, and the affected individuals may become asymptomatic without treatment. The hypothetical dividing line, drawn in Fig. 3, separates the recognisable clinical forms of tuberculosis from the silent ones, and could be called as the Clinical Horizon, after Gothi. (2) It could be observed that most of the infected persons remain below the clinical horizon, never manifesting disease. Even when clinical forms develop and the line showing the course of clinical events is traced to be crossing the clinical horizon, the dominant host factor supervenes and **there is at all times, an over-riding possibility of self-cure.** This has been observed even in pulmonary tuberculosis, as its natural dynamics, both during the pre-chemotherapeutic days, as well as during the longitudinal surveys carried out by the National Tuberculosis Institute, (NTI) Bangalore.(2)

The self-limiting predilection for the curve of disease development in the course of its natural dynamics, as observed both in animal models as also in human hosts, finds an almost synonymous replay in the epidemic curve of TB in the community as well !

⑪. The course of TB epidemic

In Fig. 4, the hypothetical epidemic curve of tuberculosis, as adapted from Grigg (3) is depicted. The epidemic curve is essentially the same as for any other infectious disease, with an ascending limb, the peak or transitional phase, a descending limb and a steady endemic phase. The whole epidemic would last several centuries, instead of a few weeks or days, as in the case of other epidemics. The ascending limb is characterised by a proximity between the rates of infection, disease and deaths, the gap between them widening as the epidemic would progress. The ascending limb is steeper than the descending one. The former is characterised by higher rate of disease and death in the underprivileged and in those who are relatively more susceptible, e.g., the females and young ones. The epidemic curve in the urban areas could be faster to develop, with higher rates than in the rural, because of higher transmission of infection taking place due to factors related to relatively more intercourse of population and destabilisation of population groups. As the epidemic would develop in time, the urban rate would start declining, even when the rural rate is going up, causing a crossover. As the epidemic curve would be ageing, the relative peaks in respect of the mortality rates, disease & infection rates would be attained one by one, followed by decline: starting with the mortality - rate – the earliest to reach the peak and start declining. (Fig. 5). **Based on the above relative behaviour pattern of indices within the hypothesis of the epidemic curve, epidemiologists would conveniently draw conclusions on the age of the epidemic in a given community.(3) (4)**

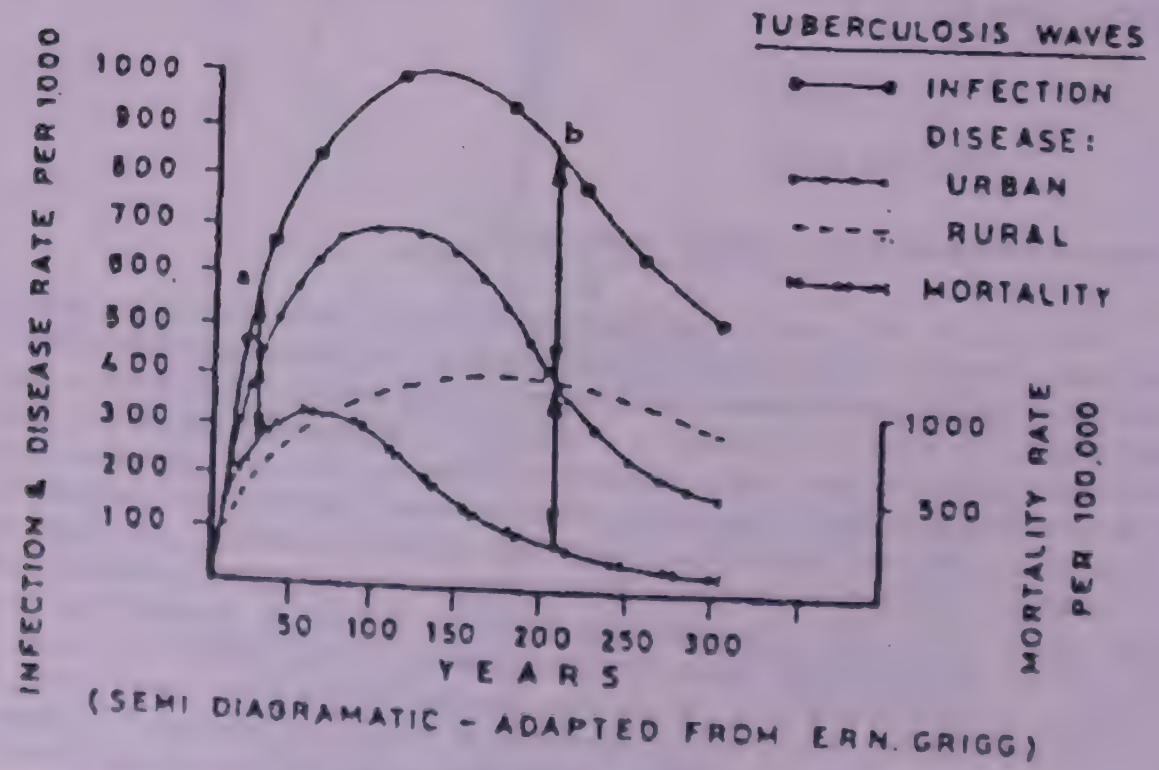


Figure 5 : Development of the wave of tuberculosis epidemic through time.
The tuberculosis epidemic curve develops through centuries. The essential proximity of infection, disease and mortality curves characterises the phase of spread (shown with arrow 'a") Wide gaps between one and the other rate develop at the peak and descending limb (shown with arrow 'b'). In India, gaps similar to the latter, exist now.
Source : Chakraborty AK [4]

It is however argued that the benefit of progress in the socio-economic sector, which has often been ascribed to be the factor principally guiding the course of the epidemic, even without active intervention, have been very "unequally distributed both between and within nations." (5) As a result, the epidemic curve may not be uniform, even within a given nation or a community. This factor of course finds an expression in Grigg's model for urban and rural curves following different courses. Because of this disparity and variable attributes of socio-economic nature within nations, it may not be possible to develop an epidemic curve for the whole nation, especially with those with large population sizes, as the disease behaviour in groups within it may not after all be uniform. It is argued that whilst it may be appropriate to conceive of tuberculosis epidemic curves for the developed nations ("industrialized nations"), it may not be so for the others. In the latter, the transition from one to the other limb of the curve may not, after all, be uni-directional, as envisaged. Progress could also be followed by "counter-transition" in the epidemic, in case there is lack of sustained direction of socio-economic transition over a long period of time, say over centuries. In the case of a long sustained phase of economic reversal, as is happening in some of the sub - Saharan countries, there could even be several epidemics curves superimposed on one another.

Grigg's model of course provides for small reversals of trends following the situations of deprivations triggered by war/pestilences, as seen in Europe during the world – war years. Past these temporary aberrations, the course of the natural trend is resumed. Grigg terms the former as cyclic, and the long term one, secure from temporary fluctuations, as the "secular curve". Of course it is possible to envisage rapid socio-economic changes, as in Japan, hastening the epidemic transitions in time rather drastically. In such an instance, the progress of the epidemic curve could also be squeezed in time, one phase dovetailing other in rather rapid succession (accelerated model)(5). However, in such a contingency, the decline could no longer be as steep, after a time, as seen in the classical European model.

It is pertinent to emphasise here the fact that even within the group of industrialised countries, the epidemic curves could be of different ages, for example the decline in England and Wales appeared to have accelerated ten years later (1950 onwards) than in the Netherlands. (6) Even within a developed country, the trend among the outside born group was different than in those indigenously born.

For the purpose of this presentation, the hypothesis put forward by Grigg is generally followed. However, it is conceded that for countries like India, China etc., because of the large masses of people with socio-economic disparity within them, a single epidemic curve of tuberculosis may be too much of simplification for a hypothesis !

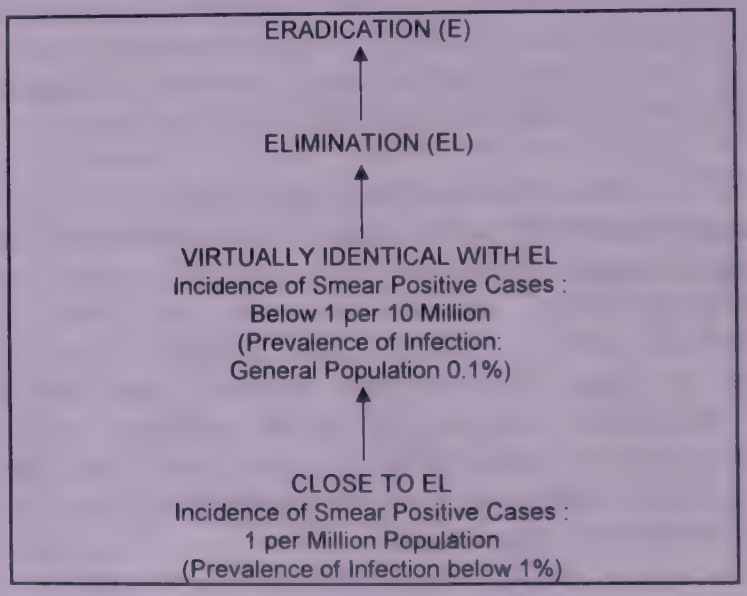
IV. Epidemiological trends by regions in the world

The countries in the world could be seen to form themselves into two broad groups, in terms of the hypothesis made by Wade Hampton Frost, way back in 1940, i.e., those in whom the "tubercle bacilli is losing ground", so that a given number of sputum positive transmitters "do not succeed in establishing an equivalent number to carry on the succession" , and the others, in whom "no such prospect is in sight" in the conceivable future. (7) It is another matter that these two groups also enjoy an equivalent economic grouping (i.e., the industrialised and the developing nations) ! Apart from these two major groups, there are other countries, positioned intermediately, in whom the

tuberculosis situation may not be at these two extremes. It could therefore be convenient to consider the countries in the world into, say, four epidemiologic groups, given their current trend and future prospects. (8)

Though Frost had talked of an eventual eradication, the goal of anti- tuberculosis measures could obviously be less optimistic. Instead of "eradication" or "control", the pragmatists, on the basis of actual observation of the situation in the past and projecting it to a conceivable future in respect of the Netherlands, would like to see the **"goal" defined in more precise terms, as : "Elimination", "Virtual Elimination" and "Close to Elimination".** Table 1 gives the definitions used and Table 2 the classification of the countries, going by these definitions. (8)

Table 1 : GLOBAL TUBERCULOSIS SITUATION – 1
(SUGGESTED DEFINITION OF GOAL)



Source : Styblo K. [8]

Table 2 : GLOBAL TUBERCULOSIS SITUATION – 2
(GROUPING OF COUNTRIES)

Annual Risk of Infection (ARI)	Annual Decline	Group
0.1 – 0.01%	≥ 10%	GROUP I Industrialised Countries (Netherlands, Norway, etc.)
0.5 – 1.5 %	5 – 10%	GROUP II Middle Income Countries (Latin America, West & North Africa, etc.)
1 – 2.5%	≤ 5%	GROUP IIB Middle Income Countries (East & South East Asia, etc.)
1 – 2.5%	0 – 3 %	GROUP III (Sub-Saharan Africa and Indian Sub-Continent, etc.)

Source : Styblo K. [8]

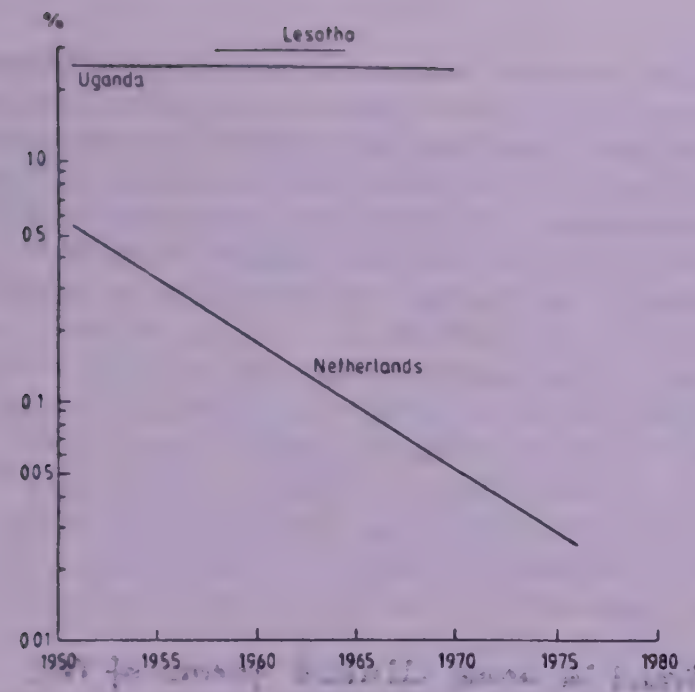


Figure 6 : The annual risk of tuberculosis infection in the Netherlands, Uganda and Lesotho.
Source : Styblo K [9]

Table 3 : INDIAN SITUATION SET AGAINST THAT IN THE EPIDEMIOLOGICALLY ADVANCED COUNTRIES HEADING TOWARDS THE GOAL OF `ELIMINATION'

Country	Epidemiological Situation			
	Present		Qualification for `Close to Elimination' Status	
	Incidence of smear positive cases per million/year	Prevalence of infection all ages (%)	Incidence of smear positive cases per million/year	Prevalence of infection all ages (%)
Most Advanced *	12 – 15	15	} 10 (1.2)	1 0
India **	500 (750)	40		

* Norway, Netherlands, etc.: (ARI 0.1 to 0.01%, 10% Annual Decline, `Close to Elimination' Status projected to be achieved by 2025 A.D.)
NOTE: ** Figures base on NTI Survey. [4] ( ) Calculated for 1.5 million population i.e., an average Indian district

Table 4 : The Global toll of tuberculosis.

Region	People infected (millions)	New cases	Deaths
Africa	171	1 400 000	660 000
Americas*	117	560 000	220 000
Eastern Mediterranean	52	594 000	160 000
South-East Asia	426	2 480 000	940 000
Western Pacific ◆	574	2 560 000	890 000
Europe & other industrialised ¶ countries	382	410 000	40 000
Total	1 722	8 004 000	2 910 000

* Excluding USA and Canada
◆ Excluding Japan, Australia and New Zealand
¶ USA, Japan, Australia and New Zealand.
Source : Kochi A. [10]

The epidemiological trend of tuberculosis, in the best possible scenario, and those in the worst, is depicted in Fig. 6 by the respective risk of infection in these countries (9) (also refer Appendix I).Table 3 gives what these would mean in terms of the comparative incidence of smear positive cases in India and Group I countries, as at present and when the goal of close to elimination is to be addressed ! (4) In respect of the risk of infection, there is an exponential decline (upto about 14% annually : some 5% of it natural and the rest attributed to anti-tuberculosis measures in specific) in the countries with the best possible scenario (example, The Netherlands). For the worst, on the other hand, (example, Lesotho), there is nil or minimal decline of between 1 & 1.4% annually. In the former the problem is likely to be halved in 5 years without any such prospect for the latter. In terms of cases on the other hand, the annual incidence of 12–15, as at present, remains to be reduced to 1-2 for 1.5 million population in the group I countries, in order to achieve close to elimination status by the year 2025. **For India, on the other hand, this would mean the present incidence of smear positive cases of about 750 annually in the same population (i.e., equivalent to an Indian district), requiring to be brought down to 1-2 by that date. (4)**

V. Global toll & trend in some clinical forms of TB,

Mortality :

Tuberculosis appears to be the most leading cause of death from a single pathogen globally. In 1990, the largest number of deaths had occurred in the SE Asian region (940,000), followed by that in the Western Pacific Region (890,000) and African region (660,000) (Table 4). (10).

It is estimated that annually about 40,000 deaths occur in all the industrialised countries, put together. The data by regions in the world is given in Table 4.

The overall conclusion with respect to the trend in mortality rates is that the mean age of tuberculosis deaths increases, as the Annual Risk of Infection ARI declines. (11) The number of deaths in children would decline faster than the ARI. The reason for the former observation is that with time, tuberculosis cases tend to get concentrated in the adult age groups.

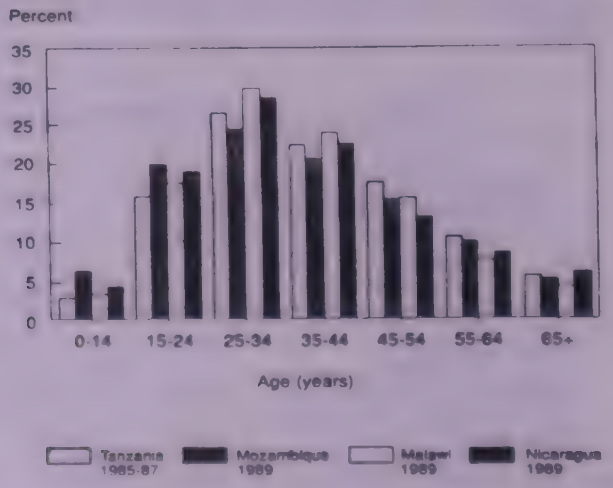


Figure 7 Age distribution of smear-positive tuberculosis in four Sub Saharan Tuberculosis Programmes
Source Murray C et al [11]

Smear Positive cases :

Fig. 7 shows the age distribution of smear positive tuberculosis, out of total incidence of tuberculosis in some African countries (developing countries). (11) Clearly, the diagnosis of smear positive cases is relatively rare in children. **Smear positive cases are concentrated more in adults**, more than 80% of these occurring between the ages 15 – 54 years. There is a relative concentration of cases in the age groups of 25 – 34 and 35 - 54 years, the peak being in the former. (11) The situation among the industrialised countries appears to be quite different. In 1990, for example, 70% of the cases in Sweden, appearing among the Swedish-born, was in the age group of 65 + years. In England and Wales also, as in many countries of the Western world, the highest notification of cases was in the age group 65 + years. (12) Even within the same country, the concentration of cases appeared to be related to ethnicity (or, socio-economic parameters). The age – distribution of cases in the non-Hispanic Whites, being distinctly different from those in the minorities in the United States, could serve as an example. (Fig. 8) (13) The relatively unfavourable epidemiological scenario prevailing among the American minorities, as brought out in the 24 year Williamson county study, (14) remains essentially the same, the socio-economic progress in the US, in the meantime, notwithstanding !

Figure 8 : Age distribution of reported tuberculosis cases, by race and ethnicity, United States, 1985.
Source : Rieder HL et al [13]

Other forms of tuberculosis :

From the analysis of data in USA and the African countries, it was estimated by Murray, that there could be 1.22 cases of smear negative and extra pulmonary forms of tuberculosis for every case of smear positive tuberculosis. (11)

VI : Tuberculosis in India

Case rate : Information from tuberculosis case surveys in India, carried out since 1959, including four longitudinal surveys in various areas namely, Delhi (urban area), Madanapalli, Bangalore and Tamilnadu, has revealed no change over a period of over 35 years. (4). The first two had infact included efficient treatment services provided in the area. The last one also had provision for treating every identified case, through the National TB Programme, operating with its given efficiency. The Bangalore area survey, on the other hand, was planned to study the natural dynamics, without a programme in the area. It was intended to study the baseline dynamics, to assess intervention effects at a later date, with a programme introduced. At the time these surveys were planned, it was logical to expect tuberculosis case prevalence rate to be reduced over a period of time, following intervention. However, it is understood now, that prevalence and incidence of cases are not affected over relatively short periods of time, unless very intensive treatment of smear positive cases are carried out, especially true for the countries with a high infection transmission. Moreover **small rates of change in the case prevalence rates, which are already small, could not be appreciated in sample sizes,** not specifically decided upon to appreciate very small change in them. (4)

From the natural dynamics of tuberculosis, as studied in the rural area around Bangalore, the tuberculosis situation is supposed to be presenting a steady state. (Fig. 9) (4) Without active intervention, a third of the existing pool of bacillary cases in a year would get eliminated, through death and natural cure. But during the interval, the same proportion gets added to it. The mathematical model constructed at the NTI, feeding the dynamics of death and transfers, observed over 5 years in various identified groups, by diagnostic criteria adopted at the NTI survey, showed that even in 50 years, tuberculosis case rates would come down only minimally. (15) Very large population sizes would be required to be surveyed repeatedly to appreciate a change, if any, which rules out the possibility. (Table 5) Various case finding and treatment levels were input into the model, as per the data available from a study on programme dynamics. (16)The model demonstrated that high levels of intervention, however, could result in substantial change in the prevalence rates (Fig. 10). (15)

Table 5 * : POPULATION REQUIRED FOR REPEAT SURVEYS TO VALIDATE CASE PREVALENCE RATES DERIVED FROM EPIDEMETRIC MODEL UNDER SOME INPUT VARIABLES OF INTERVENTION

(Given Initial Case Prevalence Rate: P = 0.389%)
(For Confidence Level 95%, One Sided Test, Power 80%)

Input Variables of Intervention						
	(a)		(b)		(c)	
Year of case observation	Case `P' rate estimate from model (%)	Popn. required in a survey for validation	Case `P' rate estimate from model (%)	Popn. required in a survey for validation	Case `P' rate estimate from model (%)	Popn. required in a survey for validation
10	0.312	89,478	0.281	43,609	0.281	43,609
25	0.286	48,302	0.239	21,194	0.238	20,881
50	0.255	27,232	0.190	11,104	0.175	9,354

Input Variable of Intervention : a) No intervention – 50 years ; b) No intervention – 5 years ; 33% case-finding efficiency (CF) and all cases on standard regimen (SR) for 6-50 years; c) No intervention – 5 years; 33% CF and all cases on SR for 6-25 years; 33% CF – 20% cases on SCC; 80% on SR for 26-35 years; 40% CF – 40% on SCC and 60% on SR for 36-50 years.

* Calculated on declining prevalence rates of cases (initially by 3.8% per year, decelerating with time, as per model)

Source: Balasangameshwara, Chakraborty, Chowdhury [15]

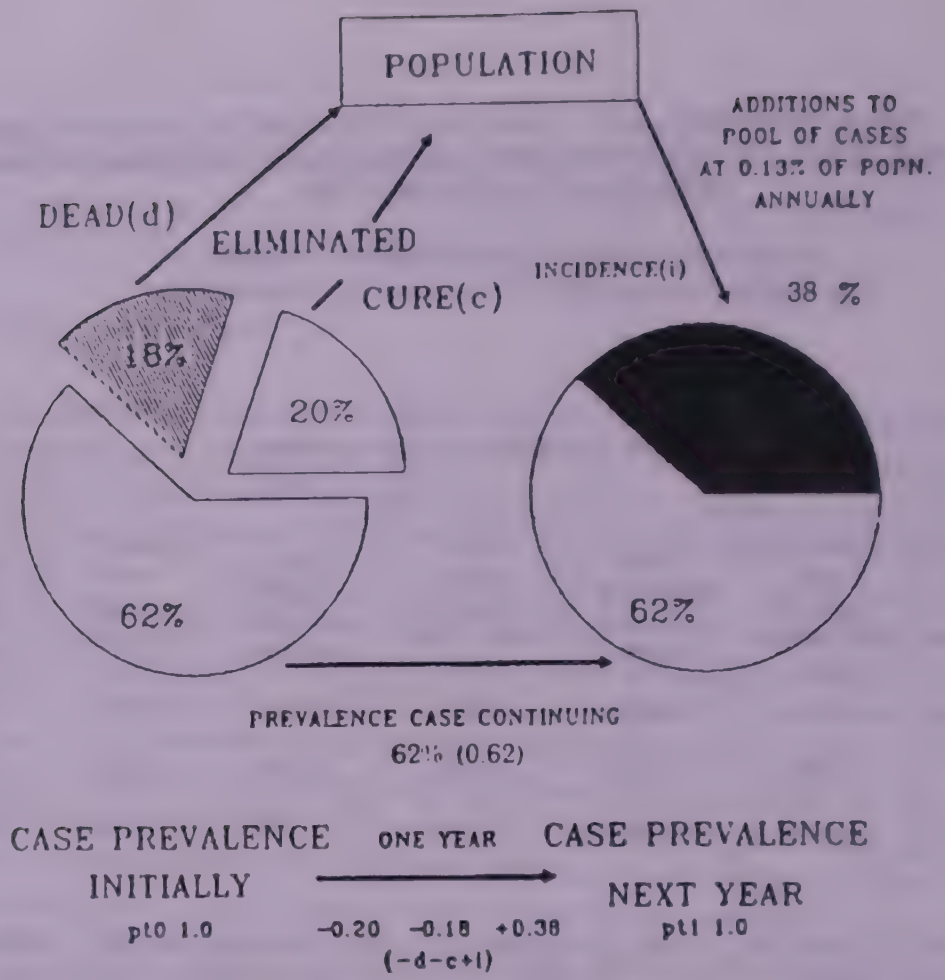


Figure 9 : Pool of tuberculosis cases in the community. (Natural Dynamics)
Source : Chakraborty AK [17]

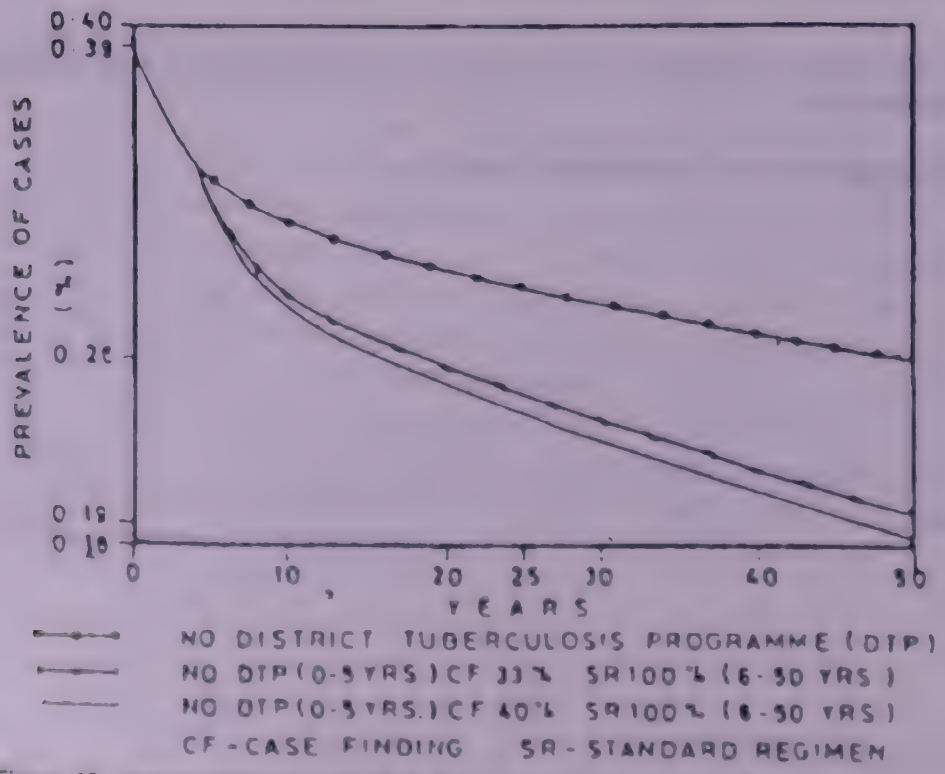


Figure 10 Model depicting hypothetical time-trend of tuberculosis in Bangalore rural area
Source Chakraborty AK [17]

ARI :

It could be observed from the data from various infection surveys carried out in India, that the ARI had varied from area to area and was in the range between 1.0 & 1.2% per year (17) (Fig 11). Even though tuberculosis case trend showed no change in any part of the country, annual risk of infection measured over a period of 23 years had registered an annual decline at about 2% per year in Bangalore rural areas. In the other area, where the ARI could be studied over time, there was however no decline in a series of surveys by the Tuberculosis Research Centre, Chennai, in Tamil Nadu. (17)

Epidemic Curve in India:

Over comparatively shorter period of observation of the natural dynamics in the NTI-longitudinal survey or the TRC study, tuberculosis appears to be having a steady state in India. Evidences are available to permit one to draw the hypothesis that the epidemic situation in India is probably on a slow downward curve of the epidemic.(4) Such evidences could be as follows : declining mortality and case fatality rates due to TB, decline in meningeal and miliary forms of the disease, relatively high prevalence of cases in higher ages with a low rate of positive cases in children, relative concentration of cases in higher ages, higher prevalence of cases in the males, especially adult males and equal prevalence rates across the urban-rural divide. **However, even if on a downward limb of the epidemic curve, the decline at present could only be minimal, as seen from direct measurement of ARI.**

It could be concluded that India has the epidemiological trend in common with the countries of the sub-Saharan region, having an ARI between 1 & 3% and an annual decline in it, varying between 0 & 3%. Only when high efficiency intervention, both in case finding and treatment is carried out, would a decline of between 7-10% result. It is suggested that suitable ARI-studies are designed to obtain a trend in the tuberculosis situation, subject to various levels of the intervention – efficiencies. Small population sizes, as required for infection surveys, make such studies possible to conduct and they could yield valuable information. (17)

The tuberculosis burden in India :

The likely estimates on the tuberculosis problem in India expressed both as average rates (with range for 95% confidence intervals) and the absolute numbers are given in Table 6. (17) The Table also incorporates rates, after adjusting for under and over diagnosis made in the surveys, from which the rates are derived, depending on the survey method used. (17) It could be of considerable significance to note that the adjusted rates for culture positive cases could be 6.0 per thousand and that for radiologically positive cases, about 3.0 per thousand, on the average. The rates are recommended to be used for estimation of the burden, instead of the currently used rates of 4 & 16 respectively. **When thus adjusted, the average rate of pulmonary tuberculosis could be 9.0 per thousand, instead of the current 20.0 per thousand for the country on the whole.** These rates have recently been discussed and accepted at the workshop on country-specific estimates on TB morbidity and mortality organised by the WHO SEARO at New Delhi in November 1996.

Table 6 : PROBLEM OF TUBERCULOSIS IN INDIA (AVERAGE)
(estimated on 1991 population)

1.	Population : 844 million : 726 million in 5+ age (85%)	
2.	Prevalence of Infection	
	a) Rate – 38% (all ages)	b) more than 50% in 40+ age
3.	Prevalence of radiologically active abacillary pulmonary tuberculosis	
	a) Rate – 1.6% (0.3, 0.26 - 0.47%)	b) Number – 12 million (2.3 million)
4.	Prevalence of positive cases	
	a) Rate – 0.4% (0.6, 0.3 – 1.1%)	b) Number – 3 million (4.4 million)
5.	Prevalence of total cases	
	a) Rate – 2.0% (0.9, 0.56 – 1.57%)	b) Number – 15 million (6.5 million)
6.	New patients arising annually : 2.5 to 3 million	
7.	New bacillary cases arising annually : a) Rate – 0.13%	b) Number – 1 million
8.	Case fatality rate : 14% in untreated bacillary cases	
9.	Mortality (annual) : a) Rate – 50 – 80/100,000 population (45, 28-71 per 100,000) *	b) Number – 0.3 – 0.5 millions (0.42, 0.26 – 0.67 millions) *

Rates in bracket based on Appendix Table II
* Derived from WHOSEARO workshop on country specific tuberculosis estimate, 1996 (unpublished)
Source : Chakraborty AK [17]

VII : Epidemiological indices for appreciating trend in tuberculosis

Mortality & Case Fatality :

A discussion on the likely indices required to be studied to understand the trend of tuberculosis in a community will not be out of place here. As discussed earlier from Grigg's work, tuberculosis mortality (or case fatality) is the earliest index to register a decline in an epidemic curve. Moreover it is very responsive to treatment, even to inadequate treatment. However reduction in case fatality does not represent a change in transmission and is therefore not really indicative of the tuberculosis trend. No doubt, as discussed earlier, the shift in mortality due to tuberculosis to older age groups could give an indirect evidence of the epidemic state.

It is generally recognised that for appreciation of change with time, culture positive case prevalence rate of tuberculosis is not the appropriate index to study. In the Kolin study, for example, the culture positive prevalence rate had been observed to register a high only during the survey years. (18) It was the smear positive case rate (also positive by culture) which was independent of the effect of case detection surveys (Fig. 12). However, smear positive cases, detected in a survey, and not confirmed on culture, were mostly found not to be the real cases. (19) Thus prevalence of smear positivity, unless supported by culture, is not representative. At the same time, prevalence of real

smear positive cases, in situations where there is considerable pooling of untreated or inadequately treated cases, is the index which is influenced in an effective control programme. For example, in a situation like as it is in India, where prevalence is thrice the annual incidence, an effective control programme could effectively bring down the case prevalence, till probably the point when incidence and prevalence are in the same proportions in the community (1:1). **Prevalence of real smear positive cases is likely to be a good epidemiological index, when the intervention measure is either very effective (close to 100%) or when there is no treatment at all. (18) It is understood that inefficient treatment services only multiply the prevalence of smear positive cases, due to pooling of inadequately converted cases.**

Incidence :

Incidence rates of cases would not undergo any change following the best of interventions, in a comparative short span of 30-40 years, given the long span of the epidemic. The incidence cases result from the breakdown among the previously infected decades back, and the incidence rates are therefore constant year to year. In any case, these represent the transmission situation existing in the community decades ago.

ARI :

It should be understood that **incidence of infection in the younger age group can really be the index representing the current transmission situation.** For studying the incidence of infection directly, repeat surveys need to be conducted in the same population, testing the same persons twice. However from figures of prevalence of infection, Annual Risk of Infection (ARI) could be worked out using a model. (18) A series of tuberculosis infection surveys, carried out at intervals of 7-10 years, depending on and related to the intervention-efficiency in a given area, could give a trend, following intervention. As already stated, Styblo had demonstrated in one of the industrialised countries (The Netherlands), about 9% change per year attributed to the intervention measures, over and above about 5% natural decline (i.e, a total of 14% or so annually). It is suggested that for developing countries, the natural decline of 0-2% may have to be augmented to be between 5-10% following an intervention, for it to be cost effective.

It is possible to work out appropriate sample size of population with the hypothesis of decline given above, to measure likely change in India or elsewhere in the developing world, making allowance for the Design Factor (say between 2 & 3), confidence intervals of the proportions in the population studied (95%), years intervening the surveys (say 7 to 10 years), relative proportions of annual change designed to be appreciated (say, upwards of 50% in 7 years) and relative precision of the estimates. (20), (21) Given the proportion of the BCG vaccinated in children, this is also a variable to be considered in deciding the sample size.

Risk of infection in vaccinated subjects:

It is understood that ARI is studied among the unvaccinated subjects only.(18) However, in situations where mass BCG vaccination at birth or soon after is the national

policy, it is not a convenient sample to have, as most of the children will already be vaccinated. The alternative could be to study the infection risks in the vaccinated. Studies are required, on the lines as carried out originally by Raj Narain, for identifying the newly infected subjects by the Differences of Reactions Method. (22)

Estimates from Notification data :

In view of the paucity of data from recent surveys and widespread use of BCG world-wide, estimates of clinical tuberculosis based on ARI-data is found to be of limited use. Dolin has attempted to estimate global incidence from notification data, routinely available from health-services reporting. (23) Notification data were considered reliable when provided by programmes with an established surveillance system. In most developing nations, it could again be unreliable in the absence of the latter. In such situations the best possible estimate from a neighbouring country was applied. To have a new set of country-specific data on incidence and mortality, together with their projections on to the future, would need models on the lines followed by Dolin et al. (23) **Huge costs involved in obtaining incidence rates by conducting population surveys, could be avoided, if the routine data could be interpreted, after necessary adjustments.** Studies are called for in order to understand the problems in the use of such data, keeping in view their contextual modernity.

The estimates on disease burden and toll, as presently being calculated from, however, they may lack the accuracy necessary for trend-calculations, especially to measure intervention-effects.

VIII: Threat perceptions from HIV

It was Zaki, in a paper in 1968, who had commented that "the international tuberculosis situation is complicated by the growing impression that tuberculosis is no longer a major public health problem." (24) Notwithstanding the self-limiting nature of the growth of bacilli in hosts and that of disease in individuals following infection, the threat due to tuberculosis was, according to him, ever present. This was due to several factors as listed by Zaki, namely : chronicity, ability of bacilli to stay alive in body for years, the slowing decline in many industrialised countries e.g. in Japan, increased expectancy of life, high level of edemicity in ethnic groups even in the midst of affluence in the western world, the recent frequent occurrence of epidemics and the emergence of Multi-drug-resistant-tuberculosis (MDRTB).

In the course of decades, his words tend to prove true today. HIV infection, in the meantime, has come as a detrimental factor, lowering the CMI-barrier in hosts, as described earlier. Apathy of public policy makers even in many industrialised countries, e.g., that in New York City, had complicated the problem too, as was forewarned by Zaki.

Presently, less than 5% of cases of tuberculosis throughout the world are associated with HIV infection and the majority of these cases are concentrated in only sub-Saharan African countries. (10) In these countries upto 100% increase had been registered (as in 1991) in new cases of TB, posing serious problems to the resources and mechanism of the TB control operations in these countries. More stigma could be enshrouding the diagnosis of TB due to its HIV association in course of time. A higher mortality rate among HIV related tuberculosis patients could take an ominous toll. There is also an upswing of TB cases among the foreign-born in the industrialised countries. (12) even though it is generally believed that the elimination goals, discussed elsewhere in this paper, will not sufficiently be jeopardised for these countries by HIV. (9) Whereas the HIV-impact is not yet measured in terms of the epidemic trend of tuberculosis in this country, urban hospital groups in some cities have started reporting on a higher and higher association. (25) **The problem of continuing the present policy of BCG vaccination, in countries with a high prevalence of AIDS & HIV infection, needs to be looked into**, in view of the chance that the vaccine bacillus may harm HIV infected children. (9)

X. : Comments on the direction of epidemiology research in India

Some of the more pertinent epidemiological issues for investigation, as raised during the course of this paper and are particularly relevant to India, could be highlighted as follows:

a) Problem Estimation :

It is considered worthwhile to study the reliability of data obtained from reporting activity under the health services system (or the NTP / RNTCP, as the case may be). It would facilitate the development of correction factors (which could be different, from region to region, within the country) for the estimates derived from such data, to arrive at the 'true' incidence (and prevalence) of transmitters in the community (sputum smear positive). Estimates on mortality also could be made from the available data on reporting. The study could identify (and quantify) variables affecting the conversion of the reported figures into occurrences in the community (hypothetically, for example, these could be : awareness on TB among the prevalent chest symptomatics, action taking level among them, distribution of health facilities and extent of their networking, diagnostic efficiency of these, image of the service-providers, quality of reporting and its coverage by the health facilities and finally the private-public mix in service provision and reporting, etc.).

To start with, the study could be started in an area, for which recent epidemiological data is available and NTP is in place (suggest Raichur district in Karnataka).

Figures on the load of transmitters, could be used for planning, resources allocation as well as for estimating efficiency of programme-delivery (number diagnosed out of expected, number cured out of likely load, etc.).

Measurement of Trend :

a: ARI Studies :

Studies to observe the incidence of infection directly could be time-consuming and expensive, requiring at least two consecutive surveys. Consequently, figures obtained from infection prevalence, expressed as ARI, after the model of Styblo and Sutherland were found useful. (18) ARI could give the extent of different forms of clinical tuberculosis (including smear positive cases) as well. (6) Studies by the present author have shown that in India, incidence of infection in children and the ARI (as calculated) in them, ran close to each other in a period of 2 - 3 years of observation, in a sufficiently large population. (4) However, ARI could be varying from area to area in this country and would depend on the socio-economic variables, as well as on the efficiency of intervention.

It is therefore proposed that studies on ARI could be carried out in India on cluster samples of children (say 0-9 years), keeping the above mentioned variables in view, in order to test a suitable hypothesis of change, following the minimum effect of specific intervention i.e., NTP/RNTCP ("Effect Evaluation"). Areas in India could be grouped to represent some cities and the rest, in the form of a few zones, based on their respective socio-economic state of development and the likely programme-efficiency. Even within the zones, the selection of study areas could be made on the basis of stratification by an available hypothesis on the state of development (e.g., tribal areas, distance from the city, distance from district headquarter, and other known criteria). There could be provisions to repeat these studies after a minimum of say, 7-10 year period.

b: ARI and clinical disease :

Styblo had worked out a mathematical relationship between ARI and incidence of sputum smear-positive cases in the community, which is widely used at present, by the programme authorities. (11) In the Indian context, it is shown that this correlation did exist, but only for the first of the six longitudinal surveys carried out by the NTI. (17) In view of its usefulness in predicting the sputum positive load and being relatively easy for calculation, it could be useful to re-examine the hypothesis in the Indian context.

c: ARI and clinical disease in special groups :

It is possible to hypothesize that the infectors, among the population in the older age group (say 45 years and over), could have a more significant bearing on the level of transmission (incidence of infection) observed in the age group 0-9 years. This could especially be so, in view of the rather restricted mobility of the elderly, perforce remaining confined within the households, breeding a situation of supposedly close physical proximity between the infectors and the susceptibles in the community, living largely within the confines of the households! This is especially meaningful, when viewed in the light of the increasing concentration of the transmitters among the elderly, with time.

It should be possible to correlate ARI, studied in children aged 0-9 years, to the rate of transmitters (smear positive) in the older age groups. Its trend in time, as studied from NTI and TRC longitudinal surveys, could give more useful information.

d : ARI in the BCG vaccinated subjects:

Given the high proportion of the BCG-vaccinated, among children in a population, it could be investigated how to demarcate the newly infected (with *M.tuberculosis*) among those, BCG-vaccinated earlier. The method of Differences of Reactions, as followed by Raj Narain et al, could be used. (22)

- A model protocol, on (a) and (d) above, was developed by the author in association with Dr. Paul Nunn of the World Health Organisation (WHO) and Professor P. Fine of London, with WHO-assistance in 1993-94, and the same is available for reference as a working paper.

Additionally, data from the TB Prevention Trial (TRC Chennai) could possibly be analysed to correlate ARI worked out in the population without vaccination, with those among the vaccinated. (Pursuing both direct observation of incidence as well as Differences of Reactions method in each of the groups.)

c) Socio-economic variables and other disease behaviour among population covered by DOT and others in RNTCP areas :

With the RNTCP being implemented in increasingly more areas in India with time, and its working pattern of stressing on delivering DOT specifically to patients identified as likely for complying with the prescribed treatment, directly supervised, proportion of patients are being identified as potential "non compliers" and are otherwise treated. The latter could be having a different behaviour and risk pattern. They are also likely to have a different result of treatment. Either or both of these could influence the "Effect" of intervention in a given area. The problem could be more so in urban areas, but rural areas may also not be exempt from the likely trend. This differential needs to be studied, especially if the other-treated group also hails from the socio-economically unprivileged sections, without a verifiable address and migratory in nature.

D) HIV and MDRTB complicating tuberculosis situation :

The HIV coexistence with tuberculosis and the nascent problem of multi-drug resistant tuberculosis (MDRTB) in the population require special monitoring. These subjects are left to the concerned scientists to deliberate upon. However, it could be mentioned in passing here, that the implications of HIV infection for a country like India, attempting to achieve a very high BCG coverage of the children, need to be studied. It appears advisable to study this aspect in suitable animal models.

: Conclusion

The world is no doubt faced with the forecasts of increasing morbidity and mortality, in the immediate future. It is estimated that 30.0 million TB deaths will occur, of which nearly 10% (2.9 million) will be attributed to HIV, during 1990-1999 period. (23) In the same period, about 88.2 million will develop tuberculosis, 8.0 million of whom attributable to HIV infection. Much of this of course is due to population growth. However the static nature of the epidemic in most of the developing world, related to socio-economic situation, contributes no less to the rise.

The natural trend of limitation of disease in humans through CMI-intervention is adversely affected, both by socio-economic situation as well as AIDS-intrusion in the developing countries. It may not be gross oversimplification to observe that clinical tuberculosis generally seems to follow two distinct trends, one in the industrialised countries and the other in the developing nations. Whereas in the former, an onward declining trend could lead to many of the nations of the former category achieving a "close to elimination" status by the year 2025, for the developing nations this could be a far cry ! No doubt, highly effective intervention in order to reduce the level of transmission could in the long run, make it possible to have epidemiologically relevant gains, even in a developing country like India (Fig. 10). However, the required managerial skills to run efficient health programmes and necessary resources for the same could be lacking, which, at the same time, are but the specific needs for running efficient programmes, especially for the countries faced with the odds ! It could even be grossly optimistic to name the tuberculosis programmes in these countries as "control" programmes, as in India, given the absence of the concept of "control" and the almost impossible task to work for achieving "elimination goals" in the foreseeable future (Table 3).

It is recognised that, in comparatively recent times, following some degree of increased global awareness and concern, internationally co-ordinated efforts at resources mobilisation are now materialising. It is therefore upto the countries to use their managerial skills to run appropriate programmes and monitor the progress in pragmatic epidemiological terms!

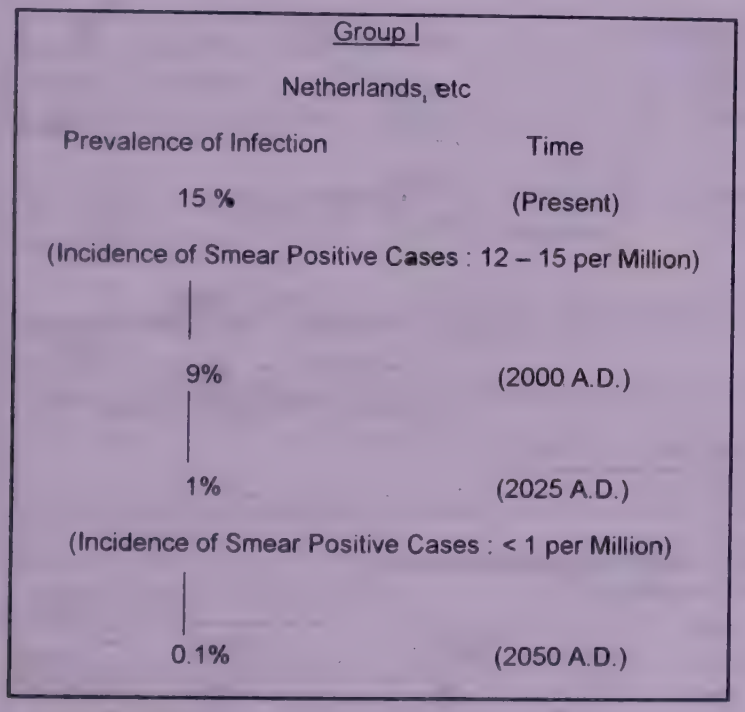
Summary

Tuberculosis, in essence, appears to be a self-limiting disease, both in animal models as well as in the human subjects. Its course could equally be self-limiting, in human population as well! For example, in the industrialised nations, tuberculosis appears to be following the rapid downward trend, not likely to be deterred even by the latest scourge, the HIV infection. By the year 2025, these countries are destined to reach a situation, which is defined as "Close to Elimination", with just one smear positive case, likely to arise in a population of one million, annually (present incidence : 12-15). In contrast, the nations with a developing economy, are currently faced with an enormous problem (say, 500 new sputum positive cases per million per year, and probably nil to an insignificant change in the risk of infection from year to year). The incidence of disease, as well as mortality rate from tuberculosis, are at a highly unacceptable level in these

countries. The absolute number of cases in fact are rising, due to population rise, as well as pooling of previous cases, as a result of inadequate treatment. New infections, like HIV would further complicate the situation, in the near future.

In this paper, the relative trends, as obtained in the countries placed in the extreme ends of the epidemiological state, are described. The problems in measurement of these are discussed. A brief reference is made to the likely investigations that could ensue in the days to come, in order to elucidate the epidemiological situation, especially in India.

Appendix Table I : GLOBAL SITUATION – TIME FRAME OF ACHIEVEMENT (BEST POSSIBLE)



Source : Styblo K. [8]

Appendix Table II : SUGGESTED REVISION OF AVERAGE TUBERCULOSIS RATES (INDIA)

	Currently used	Correction (X)	Suggested Rate
RAD	16.0 (10.0 – 19.0)	0.2	0.3 (2.6 – 4.7)
BA	4.0 (2.0 – 8.0)	1.4	6.0 (3.0 – 11.0)
Total	20.0 (13.5 – 25.0)	---	9.0
BA / RD	1 / 4	---	2 / 1

(Rate per 1000 popn x-rayed)
RAD = Radiologically active, bacteriologically negative ; BA = Bacteriologically positive (culture)
Source : Chakraborty AK [17]

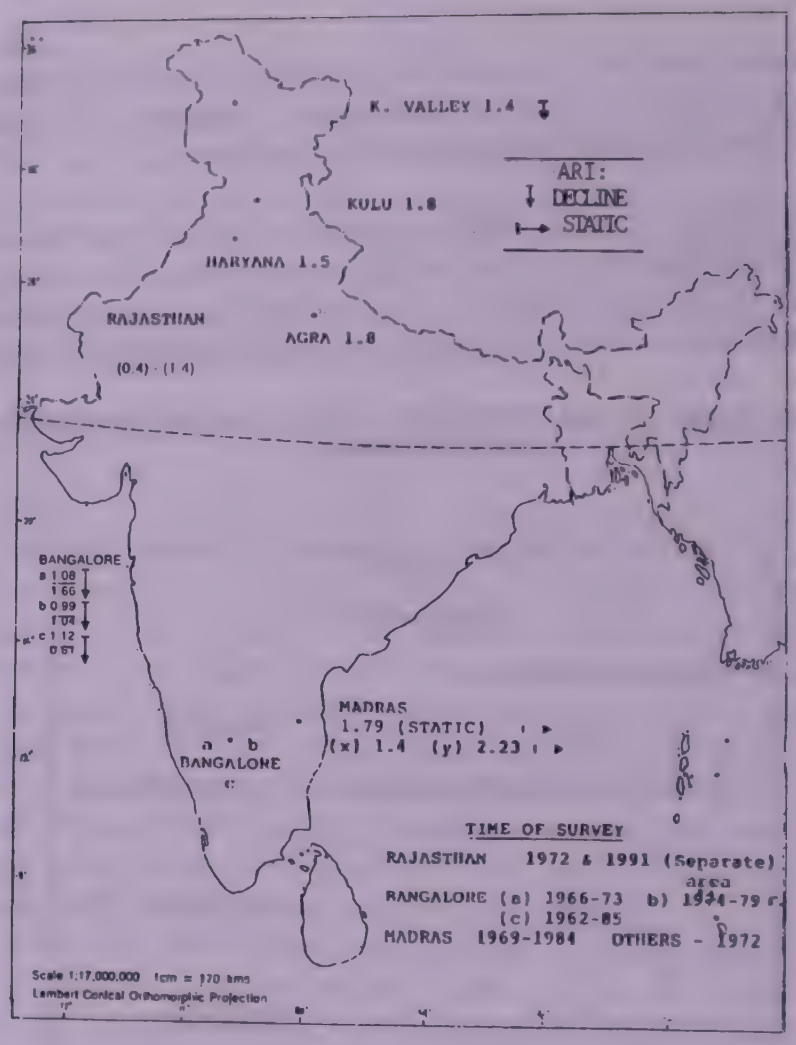


Figure 11 : Annual Risk of Infection across India.
Source : Chakraborty AK [17]

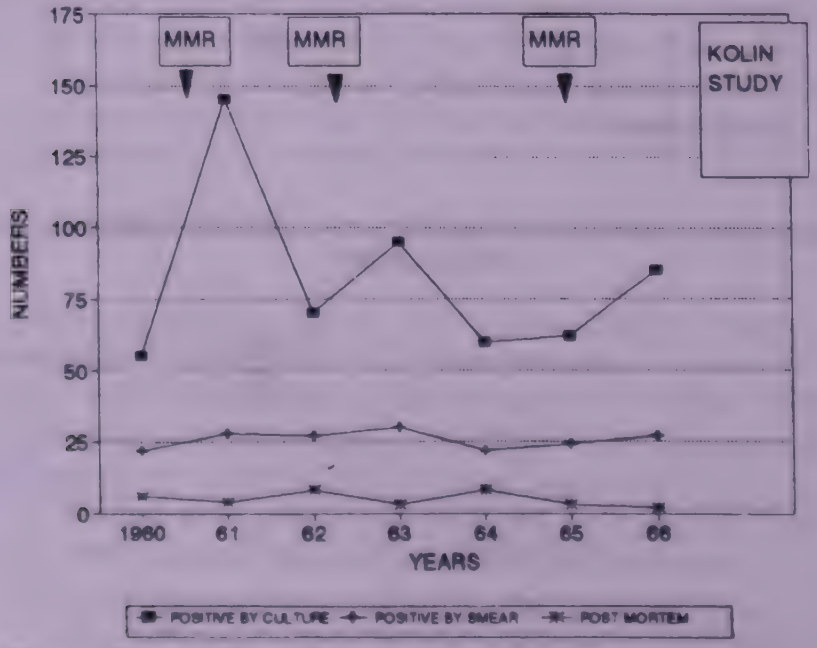


Figure 12 : Detection of bacillary TB (excl relapse) in Kolin (Czechoslovakia)
Source : Styblo K [18]

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STUDY ON ENVIRONMENTAL MYCOBACTERIA OBTAINED FROM SOUTH INDIAN BCG TRIAL AREA.

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Mycobacteria in the environment

Non Tuberculous Mycobacteria (NTM) are widely distributed in our environment and man is being constantly exposed to these organisms by various means(1). This immunologically important contact may be involved in the modulation of immunity to tuberculosis. Prior sensitization with NTM has been considered as one of the explanations for the failure of BCG to provide protection against tuberculosis in the South Indian trial. Tuberculosis surveys using PPD-B have shown that in this area, prevalence of sensitization reaches 90% in persons by age 14(2). Identification of NTM isolates from sputum samples in this area has shown *M.avium-intracellulare* and *M.scrofulaceum* to be among the important species(3). However, the actual distribution profile of the various NTM species in the environment of this area is not known.

In order to study this, in the first phase, standardisation experiments were carried out to choose an optimum method for the isolation of mycobacteria from soil and water samples(4). In the second phase, utilising the ideal procedure thus evolved, about 150 samples each of soil and water and 75 samples of dust collected from the South Indian BCG trial area on each of two occasions, once during the summer and once after the monsoon, were processed and the mycobacteria isolated were identified. Soil and water samples from a control area in Britain with a very low level of sensitization have also been included. NTM isolates obtained from sputum samples at the same time from subjects residing in the same area were also included for the purpose of comparison(5).

The results of the study indicated that the isolates belonging to *M.avium* complex (MAC) were predominant in water, dust and sputum samples and isolates belonging to the *M.fortuitum* complex were predominant in soil samples. There were no changes in profile between January and June; however, yield from environmental samples was lower in June.

Since organisms belonging to MAC include *M.avium*, *M.intracellulare* and an unnamed third species, and *M.fortuitum* complex consists of *M.fortuitum*, *M.peregrinum*, *M.chelonae*, *M.abscessus*, the unnamed third biovar of *M.fortuitum* and the MCLO, a representative number of the isolates of both these groups were subjected to drug and heavy metal susceptibility studies to see as recorded by others whether susceptibility to these agents would be of help in comparing environmental and sputum isolates(6). However, using this procedure, we were unable to distinguish between isolates from the environment and from sputum.

Molecular characterisation of MAC

Phenotypically identified MAC isolates from different sources were further characterised using plasmid profiles(7), identification by 3 specific probes, namely, DT1, DT6(8) and LiPA(9,10), analysis of lipids using gas chromatography-mass spectrometry (GC-MS) and analysis of mycolic acid pattern using thin layer chromatography(11). The results are shown in the Table.

i. Plasmid profile

Plasmid DNA analysis of a total of 62 MAC isolates, 13 each from water and dust, 16 from soil and 18 from sputum showed that the maximum number of plasmid-carrying strains were among the non-pigmented strains from water while MAC strains from sputum yielded few plasmids. These results are in contrast to those reported for plasmids from MAC strains in earlier studies where they have been reported to be preferentially found in clinical and aerosol isolates.

ii. Mycolic acid profile:

a. GC-MS : A total of 32 MAC isolates (8 each from water and dust and 10 each from soil and sputum) were also characterised for their fatty acid composition using GC-MS. Among the dust isolates, 6 were identified as MAC, and 1 each as *M.phlei* and *M.fortuitum*. For soil, 6 were identified as MAC. Of the remaining 4 strains, 3 were identified as *M.fortuitum* and 1 as *M.xenopi*. For sputum, 6 were identified as MAC, 1 each as *M.fortuitum* and *M.xenopi* and 2 as *M.phlei*. For water, 4 were identified as MAC, 1 each as *M.gordonae* and *M.xenopi*, and 2 as *M.simiae*.

b. TLC : A total of 40 isolates (8 from water, 9 from dust, 11 from soil and 12 from sputum) were analysed for their mycolic acid pattern using TLC. All the water and dust isolates tested produced a pattern typical of MAC (type II; d-keto, w-carboxymycolate). Six isolates from soil and 8 from sputum also produced a type II pattern. Two isolates from soil and 3 from sputum produced a type D pattern while 3 isolates from soil and 1 from sputum produced a type E pattern.

iii. DNA probe:

a. LiPA: A total of 39 MAC strains (7 from dust, 9 from water, 11 from soil and 12 from sputum) were tested by the LiPA probe. A total of 16 isolates (3 from water, 5 from sputum and 8 from soil) were identified as Mycobacterium sp. other than MAC. Of the remaining 23 strains, none were identified as *M.avium* or *M.intracellulare*, 1 but identified as M.scrofulaceum and 22 as MAC intermediates. Two of the isolates, one each from soil and sputum were identified as M.malmoense by LiPA and both these isolates were identified as *M.fortuitum* by GC-MS.

b. DT1 and DT2 PCR: A total of 42 isolates were tested by 2 probes for the specific identification of *M.intracellulare* (DT1) and *M.avium*(DT6). Of these, 16 strains tested positive with DT1 (*M.intracellulare*) and none with DT6. No strain was identified as *M.avium* using these probes.

iv. 16S rRNA and PCR-REA

MAC strains that gave discrepant results, as they were DT1 positive but gave negative results by the *M.intracellulare* AccuProbe assay were subjected to a detailed molecular analysis. These included PCR-restriction enzyme analysis (PRA) of the hsp65 gene and 16S rRNA gene sequencing. The results confirmed the reported heterogeneity of *M.intracellulare* as only 32% gave PRA result compatible with published *M.intracellulare* profiles while the rest of the isolates were grouped in four previously unpublished profiles. 16S rRNA gene sequencing showed that only 42% were related to *M.intracellulare*, the rest being related to others. In conclusion, by this attempt a significant number of MAC isolates from South Indian BCG trial area which were not identified by the AccuProbe assay, (DT1 positive & DT6 negative) were characterised by PRA or 16S rRNA sequencing(12).

Thus, these results emphasis the heterogeneity of environmental mycobacteria, particularly of the MAC organisms. However, a tendency for greater agreement of the results of the various tests for isolates from water and dust was observed while there was greater discordance for the results of these tests for isolates from soil and sputum.

Immuno modulation studies in animal model

Studies on the modulation of immune response to BCG resulting from prior exposure to NTM in guinea pig models showed that oral exposure to MAI did not interfere with subsequent immune response to BCG(13). Prior exposure to NTM by subcutaneous and intradermal routes also did not interfere with the immune response to BCG in the early course of challenge infection but it appeared that immuno modulation could be taking place at the later stages of the infection(14). Some differences were observed in the immune response and modulation of immune response induced by the different strains of MAC. And also at 6 weeks after challenge, modulation of protective response resulting from BCG was observed in the animals sensitized with either MAC from soil or *M.fortuitum* from soil(15).

Thus, the results of this study suggest that certain modulation of the protective immunity due to BCG was probably taking place in animals exposed to NTM first. In populations of endemic areas of tuberculosis with high prevalence of NTM, prior exposure to NTM may have similar modulating effect over the immunity due to BCG in the later course of infection. This may explain, atleast partly, the varying efficacy of BCG seen in the different vaccination trials(16).

TABLE

Detailed characterisation using DNA probes (DT1, DT6 and LiPA), GC-MS, TLC and plasmid profiles of phenotypically identified *M.avium* complex (MAC) isolates obtained from different sources in the South Indian BCG trial area.

CODES	Origin	DT1	DT6	GC-MS	TLC	LIPA	PLASMID
MAC66	NA	ND	ND	MAC	ND	ND	N
MAC57	DUST	ND	ND	ND	ND	ND	PL
MAC47	DUST	N	N	ND	H	ND	N
MAC61	DUST	ND	ND	MAC	ND	ND	N
MAC59	DUST	ND	ND	MAC	ND	ND	N
MAC60	DUST	ND	ND	M.phlei	ND	ND	N
MAC10	DUST	+	N	MAC	H	MIC4	PL
MAC11	DUST	+	N	MAC	H	MAC(MIC1.2)	N
MAC12	DUST	N	N	MAC	H	ND	N
MAC9	DUST	+	N	MAC	H	MIC4	PL
MAC21	DUST	+	N	ND	H	M.sp	PL
MAC31	DUST	+	N	ND	H	MAC(MIC1.2)	N
MAC8	DUST	+	N	ND	H	MIC4	PL
MAC36	DUST	N	N	ND	H	MAC(MCO11)	PL
MAC35	DUST	+	N	ND	H	MIC4*	N
MAC20	DUST	+	N	M.fortuitum	H	MIC4	NA
MAC46	SOIL	N	N	ND	D	M.sp	N
MAC40	SOIL	N	N	ND	ND	ND	N
MAC53	SOIL	ND	ND	M.xenopi	ND	ND	N
MAC32	SOIL	N	N	M.fortuitum	E?	M.sp	N
MAC3	SOIL	N	N	M.fortuitum	E	M.mal	PL
MAC22	SOIL	*	N	M.fortuitum	E?	M.sp	N
MAC1	SOIL	+	N	MAC	D	M.sp	N
MAC2	SOIL	N	N	MAC	H	MAC(MIC1.2)	N
MAC18	SOIL	N	N	MAC	H	MAC(MCO11)	PL
MAC52	SOIL	ND	ND	MAC	ND	ND	PL
MAC28	SOIL	+	N	MAC	H	M.sp	N
MAC51	SOIL	ND	ND	MAC	ND	ND	N
MAC48	SOIL	N	N	ND	H	M.sp	N
MAC45	SOIL	N	N	ND	H	M.sp	PL
MAC37	SOIL	N	N	ND	H	M sp	PL
MAC65	SPUTUM	ND	ND	ND	ND	ND	N
MAC13	SPUTUM	N	N	M.fortuitum	D	M.malmoense	N
MAC24	SPUTUM	*	N	M fortuitum	D?	M malmoense	N
MAC67	SPUTUM	ND	ND	M.xenopi	ND	ND	N

CODES	Origin	DT1	DT6	GC-MS	TLC	LIPA	PLASMID
MAC62	SPUTUM	ND	ND	M.phlei	ND	ND	N
MAC64	SPUTUM	ND	ND	M.xenopi	ND	ND	N
MAC14	SPUTUM	N	N	ND	H	M.scrofulaceum	PL
MAC33	SPUTUM	N	N	MAC	H	M.scrofulaceum	PL
MAC17	SPUTUM	N	N	MAC	H	M.sp	N
MAC15	SPUTUM	N	N	ND	E?	M.sp	N
MAC16	SPUTUM	+	N	MAC	H	MAC(M.sp)	N
MAC19	SPUTUM	N	N	ND	ND	ND	N
MAC63	SPUTUM	ND	ND	MAC	ND	ND	N
MAC39	SPUTUM	RGM	N	ND	D?	M.sp	PL
MAC38	SPUTUM	+	N	ND	H	MAC	N
MAC49	SPUTUM	+	N	ND	D	M.sp	N
MAC29	SPUTUM	+	N	ND	H	MIC4	N
MAC27	SPUTUM	*	N	MAC	H	MAC(MIC1.1.b)	N
MAC43	SPUTUM	N	N	ND	H	MAC(MCO11)	N
MAC30	SPUTUM	*	N	MAC	H	MIC4*	N
MAC58	WATER	ND	ND	ND	ND	ND	N
MAC41	WATER	*	N	ND	ND	ND	N
MAC23	WATER	N	N	M.godonae	H	MIC4	PL
MAC55	WATER	ND	ND	M.simirae	ND	ND	PL
MAC56	WATER	ND	ND	M.simiae	ND	ND	PL
MAC54	WATER	ND	ND	M.xenopi	ND	ND	PL
MAC44	WATER	N	N	ND	H	MIC4	N
MAC25	WATER	N	N	MAC	H	MIC4?	N
MAC4	WATER	RGM	N	ND	H	M.sp	N
MAC50	WATER	*	N	ND	H	M.sp	N
MAC42	WATER	N	N	ND	H	M.sp	N
MAC7	WATER	+	N	MAC	H	MAC(M.sp)	PL
MAC5	WATER	+	N	ND	H	MIC4	N
MAC26	WATER	+	N	ND	H	MIC4	N
MAC6	WATER	N	N	MAC	H	MIC4*	PL
MAC34	WATER	+	N	MAC	H	M.sp	PL

NA - NOT AVAILABLE; + - POSITIVE; N - NEGATIVE; ND - NOT DONE; * - CONTAMINATED; RGM - RAPID GROWING MYCOBACTERIA; MAC - *M.avium* complex by either GC-MS (pattern typical for MAC) or LiPA (*M.avium*, *M.intracellulare* or MAC intermediates)

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THE IMPACT OF GENOMICS ON THE SEARCH FOR NOVEL TUBERCULOSIS DRUGS

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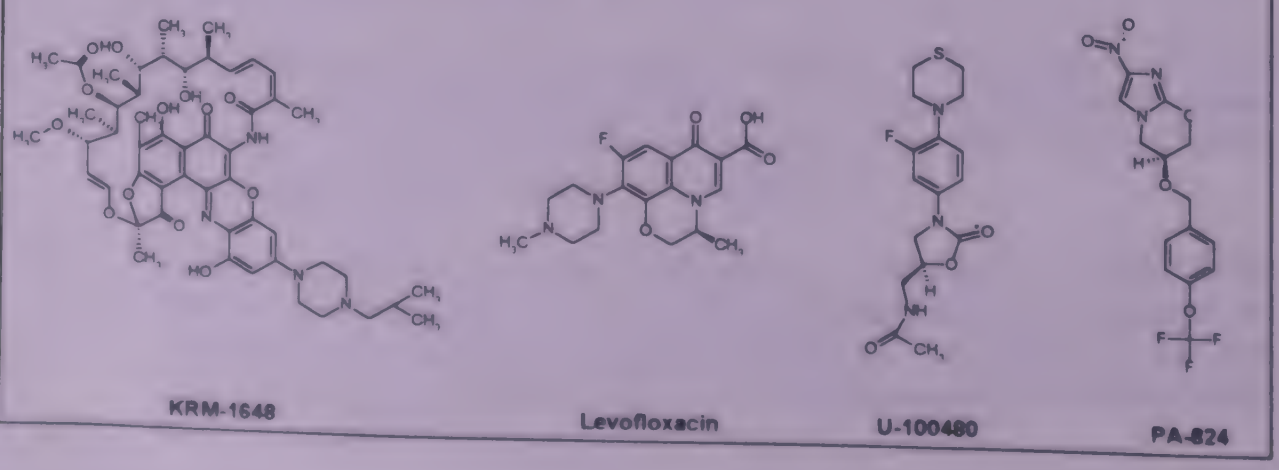
Introduction

The chemotherapeutic era of tuberculosis (TB) treatment began in 1944 with the discovery of streptomycin by Selman Waksman and his colleagues. Isoniazid, the agent with the most potent activity known against the tubercle bacillus, *Mycobacterium tuberculosis*, was synthesised in 1952. The finding that the broad-spectrum antibacterial agent rifamycin has activity against *M. tuberculosis* revolutionised treatment of TB with the subsequent development of the short-course (albeit six-month) multi-drug regimen in use today. This regimen can achieve cure rates of >95% when used correctly. Despite the wide availability of these relatively inexpensive drugs, the number of TB sufferers continues to rise world-wide, prompting the World Health Organisation (WHO) to declare TB a global emergency' in 1993.

The drawbacks of the multi-drug regimen are obvious. The number of tablets, their toxic side effects, and the long duration of the therapy lead to poor compliance. Inevitably this results in a significant rate of treatment failure, and worse, selection of resistant organisms. In a recent global survey, drug resistant TB was found in every country which reported data, with a median level of 10.4% primary resistance, and hot spots' where resistance is as high as 41% were identified (World Health Organisation 1997b). Patients with multi-drug resistant TB (MDR-TB) are very difficult to treat and continue to infect others with the resistant bacteria. In order to overcome the limitations in current therapy, the WHO recommends the implementation of a programme called Directly Observed Therapy, Short-course, or DOTS (World Health Organisation 1997a). New chemotherapeutic agents with activity against MDR-TB and drugs which can provide a shorter and simpler regimen are needed.

Figure 1 Structure of potential new TB drugs



Since the 1960's, there has been relatively little progress in TB drug development. Semi-synthetic rifamycin derivatives such as Rifabutin and Rifapentine (Baohong et al 1993) have not yet achieved widespread clinical use. The experimental benzoxazinorifamycin, KRM-1648 (Figure 1), also shows promise (Saito et al 1991, Klemens et al 1994b), but such agents represent incremental steps in therapy improvement. Although they have some advantage over parent rifampicin, such as a longer half-life in man which may permit intermittent chemotherapy, there are disadvantages in this approach. In particular, overcoming existing resistance mechanisms to the entire class of compounds can be an insurmountable challenge.

New broad-spectrum antibacterial agents that have particularly good activity against *M. tuberculosis* are also being used in the fight against TB (Figure 1). The quinolone levofloxacin (Klemens et al 1994a) is already being used in the clinic and the oxazolidinone U-100480 has good *in vitro* and *in vivo* activity (Barbachyn et al 1996).

Researchers at PathoGenesis Corporation have described a series of nitroimidazopyrans, exemplified by PA-824 (Figure 1), with potent selective antimycobacterial activity, which have no cross-resistance with other antibiotics and work via a novel, as yet uncharacterised mechanism (W.R. Baker, E.L. Keeler, S. Cai, J.A. Towell, D.R. Pastor, J.N. Morgenroth, S.W. Anderson & T.M. Arain, unpublished paper, Interscience Conference on Antimicrobial Agents and Chemotherapy, 15-18 September 1996).

Over 95% of TB sufferers are in the developing world. The drugs available today can achieve very high cure rates. *M. tuberculosis* is a slow-growing, airborne pathogen that requires specialised handling facilities and the available models of infection are lengthy and difficult. Together, these factors make developing a new TB drug a daunting challenge. In this paper, the factors that influence the direction of a TB drug development programme are discussed. The availability of the *M. tuberculosis* genome sequence adds a new dimension to our knowledge of this important pathogen, and provides greater opportunity than ever before for the rapid identification and validation of novel drug targets.

New drug profile

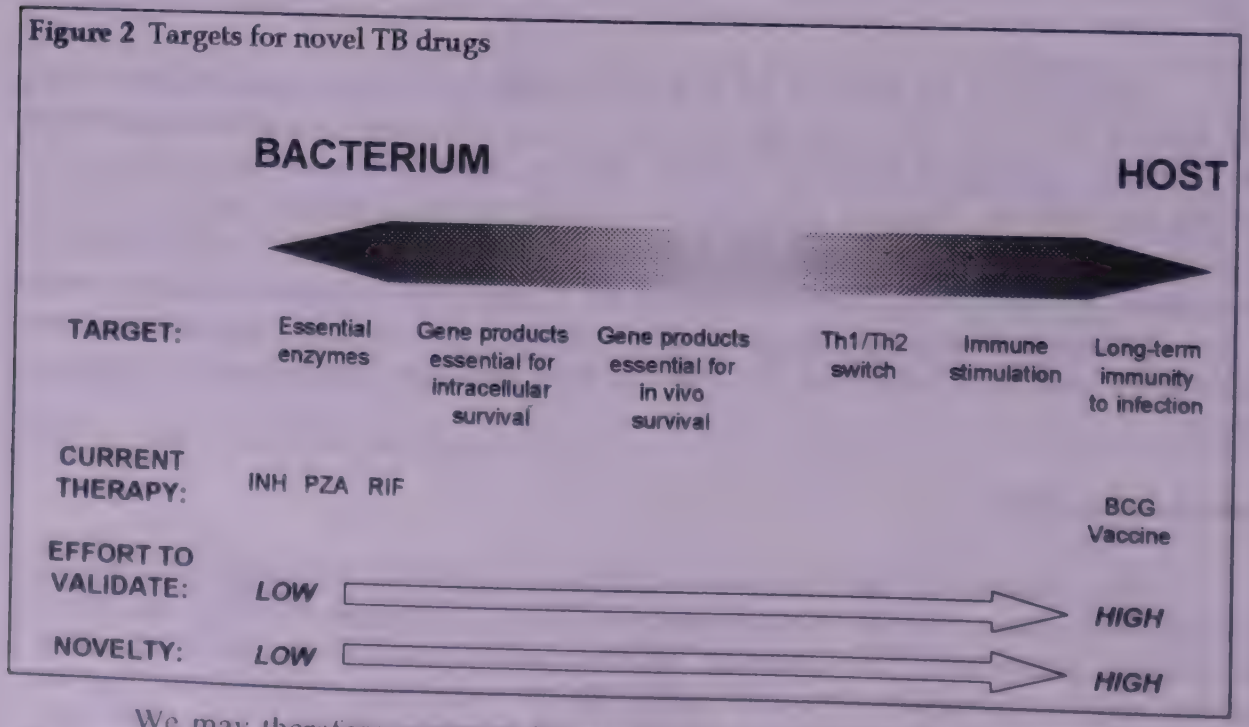
The first step in the TB drug development process is to consider the desired activity profile and properties we are aiming for, as this will determine the direction of the research programme. The 'ultimate' TB drug would possess both rapid bactericidal activity and sterilising activity, thus killing all *M. tuberculosis* populations, and the spectrum of activity would include all MDR-TB isolates. The drug must be orally bioavailable, have low toxicity, good tissue distribution to maximise activity against intracellular organisms, a long elimination half life so that intermittent chemotherapy may be considered, and last but not least, it must be inexpensive to produce given the constraints on pricing which exist. Clearly, it will be very difficult to build all these properties into a single molecule, and compromises will be made. Critically, any new drug

must offer a significant advantage over the drugs already available, such as activity against MDR-TB, better tolerability, intermittent dosing or shorter overall therapy duration.

It is most unlikely that *M. tuberculosis* has a single Achilles' Heel, and that more than one agent will always be needed to bring about a complete cure. The most achievable goal is to generate a novel bactericidal agent. Such an agent may be evaluated by the minimal inhibitory concentration (MIC) for *M. tuberculosis* growth in culture, and in short-term acute *in vivo* models of infection. Clinical testing in man is straightforward, with early bactericidal activity (Mitchison 1996) being a relatively good predictor of final clinical efficacy. On the other hand, disadvantages such as emergence of resistance, fueled by ineffective or inappropriate combinations, or reservation of the new drug for use in cases where treatment is already failing would make it uneconomic to produce such a drug, unless some other property such as a long half life would provide an advantage.

Targeting the persisting organisms offers the tantalising prospect of reducing the duration of therapy. However, specific molecular targets are not so obvious. Significant research effort is required to validate such targets and new *in vitro* and *in vivo* models must be developed if such agents are to be fully evaluated. It will be more difficult to optimise the properties of any agent that has no MIC in culture, and surrogate markers of sterilisation must be identified. Clinical trials will be lengthy and require large patient numbers, increasing development costs.

Figure 2 Targets for novel TB drugs



We may therefore envisage intervening at several steps in the disease process. The targets may be visualised on a spectrum, ranging from those functions essential for survival of the bacterium in culture through host-pathogen interaction to host immunity (Figure 2). The effort required to identify and validate targets varies considerably, with proportionally greater effort being expended on targets which have the highest novelty.

Whole-cell screening

The simplest way to find new TB drugs is to screen for whole-cell antimycobacterial activity, much in the way that streptomycin and isoniazid were discovered. Realising that it was impractical to do this with *M. tuberculosis* itself, researchers at Glaxo Wellcome employed a rapid-growing, non-pathogenic surrogate, *Mycobacterium aurum A+* (Chung et al 1995). Using this organism as a host for the *Vibrio harveyi luxAB* genes, a simple bioluminescence assay has been developed with which we can screen approximately 10,000 compounds per day which were synthesised in bead-based combinatorial libraries (G.A.C. Chung, P. Andrew, S. Polger, J. Silen, C. DeLuca-Flaherty & K. Duncan, unpublished work 1997). Other high throughput assays have been described which monitor viability with bioluminescence (Cooksey et al 1993, Arain et al 1996) or using Alamar Blue (Collins & Franzblau 1997).

Although several novel structural templates with activity against *M. tuberculosis* have been discovered this way, there are significant disadvantages to this approach. Many targets will be masked from the samples being tested, for example if they are intracellular, or are not expressed under the conditions used in the assay. In addition, it is often hard to improve upon the properties of a whole-cell active lead compound when there is no knowledge of the target. It is generally thought to be more effective to screen for agents that modulate the activity of a specific target *in vitro*, and then to further modify any leads obtained to incorporate or improve upon the whole-cell activity. For this approach to be successful, significant effort must be applied to identifying, characterising and validating appropriate targets. Targets identified by genetic means are not always suitable for screening; often the function of a gene product may not be known, or an enzyme's substrate may not be available.

Ideally, one would wish to either hit multiple targets with a single new agent, or hit a target that has a role in more than one growth phase. The advantage of the former is that it is highly unlikely that resistance will develop by a single mutation in the gene encoding the target, since the alternative target(s) would still be inhibited. Such a scenario can be envisaged for inhibition of the multiple arabinosyl transferases which recognise the substrate decaprenol-phosphoarabinose (Mikušová et al 1995, Lee et al 1995), or the three proteins which catalyse mycolyl transfer (Belisle et al 1997).

Target identification

Novel drug targets may be identified in several ways. The classical approach is to study a process, identify a protein target, and purify sufficient material to obtain sequence, then clone the gene and use reverse genetics to confirm its essentiality. It is known that disruption of cell wall synthesis is lethal to the bacterium, and several steps in this process are being analysed in this way to reveal targets. In some cases, for example arabinan biosynthesis (Mikušová et al 1995) and linkage region biosynthesis (Mikušova et al 1996), this has only progressed as far as developing a crude assay system. In other

cases, enzyme function has been correlated with a single gene product, e.g. UDP-galactopyranose mutase (Weston et al 1997) or several gene products, e.g. mycolyl transferase (Belisle et al 1997).

Alternatively, determining the mode of action of today's drugs identifies targets. For example, the *inhA* gene product was identified as a target for isoniazid (Banerjee et al 1994) and subsequently shown to possess enoyl reductase activity (Quémard et al 1995), a step in fatty acid metabolism. Biochemical studies have indicated that ethambutol acts by inhibiting arabinosyl transferase (Lee et al 1995) and further genetic studies indicated that the *emb* genes encode the target (Belanger et al 1996, Telenti et al 1997).

Progress in identifying essential genes in *M. tuberculosis* has been hampered by the lack of well-characterised mutants. The recent description of a set of temperature-sensitive mutants of *Mycobacterium smegmatis* (Belanger A, Porter JC, Hatfull G 1997 unpublished paper, ASM Conference Tuberculosis: Past, Present and Future, 8-12 July, 1997) is a step towards defining specific targets in *M. tuberculosis*. Moreover, libraries of *M. tuberculosis* mutants have been generated by randomly inserting a transposon into the chromosome, using plasmid (Pelicic et al, 1997) and mycobacteriophage (Bardarov et al, 1997) delivery systems. Although it is not possible to recover mutants generated when the transposon interrupts a gene essential for replication, we may nevertheless deduce which gene products are essential for vegetative growth by sequencing the insertion points and by referring to the genome sequence (with the proviso that there may be polar or other effects that will complicate interpretation).

Virulence gene targets

The pathogen must employ a range of virulence mechanisms that enable it to establish an infection and to survive within the host. Genes encoding virulence factors may only be expressed at certain phases of infection, and may not be required for growth on agar plates. Alternative strategies are employed to find virulence genes, in both macrophage and whole-animal models. These include IVET (In Vivo Expression Technology) (Mahan et al 1993), *in vivo*-expressed promotor trapping (F. da Silva-Tatley & M.R.W. Ehlers, unpublished work, 1997), signature tagged mutagenesis (Hensel et al 1995), RNA differential display (Fislage et al 1997), RNA arbitrarily primed PCR (McClelland et al 1995) and proteomics (see below). Although such methods are useful in their own right, they are much more powerful when the whole genome sequence is available. Only a relatively short DNA sequence need be determined in order to locate the complete gene, and furthermore, the gene may be placed into context, important when neighbouring genes may have a related function, or may be co-expressed.

Genomic approach

By having the complete sequence of the TB pathogen at our fingertips, it is self-evident that the sequence of every single target is known. The challenge is to identify

which of the approx. 4,500 open reading frames is essential under any given set of growth conditions. The first step is to build a metabolic map, by comparing the mycobacterial genes present with those genes of known function isolated from other organisms. This may provide a clue to the way that *M. tuberculosis* survives and grows. For example, the presence of genes associated with anearobiosis may suggest a strategy for killing the bacterium in the sterilising phase. Inevitably, many open reading frames in the genome sequence will have no counterpart in other bacteria, and hence it is not possible to assign a function to the gene product. Employing high throughput screening to identify antagonists is one way to validate such targets.

Comparison of the *M. tuberculosis* sequence with that of other bacteria reveals both what is in and what is not - genes uniquely found in mycobacteria represent particularly attractive targets. The unusual situation of having the complete genome sequence of two *M. tuberculosis* strains, namely the laboratory strain H37Rv (http://www.sanger.ac.uk/Projects/M_tuberculosis) and a recent clinical isolate (http://www.tigr.org), may be of benefit if we assume that the clinical strain has greater virulence in man. Differences in the intergenic regions, where gene promoters are located, may suggest clues to pathogenesis.

Proteome analysis

A complementary approach is to study the total protein complement, or proteome, of the bacterium at different stages of the growth cycle. Proteins are isolated then separated by electrophoresis in two dimensions, firstly by isoelectric focusing and thereafter by molecular mass, to yield a characteristic pattern of spots after staining (Urquhart et al, 1997). The individual protein in each spot may be isolated and identified by accurately determining its mass in a spectrometer either directly, or following trypsin digestion and sizing of the resulting fragments. Reference to a database of predicted protein molecular weights generated from the mycobacterial genome sequence would tell which protein is present in the spot. If the protein appears not to be in the predicted set, techniques such as tandem Nanoelectrospray mass spectrometry yield sequence data from which the protein may be identified. This technique is particularly useful for detecting and analysing post-translational modifications such as glycosylation (M. Ward, W. Blackstock, M-P. Gares, D.B. Young & C. Abou-Zeid, unpublished work, 1997).

In summary, the availability of the genome sequence of *M. tuberculosis* is providing many benefits in the search for new drugs to treat TB. The efficiency and speed with which targets may be identified increases the likelihood that novel potent leads will be found, which may then be developed into the next generation of anti-TB drugs. Furthermore, new approaches may be followed, thereby increasing the diversity of targets that are available for study.

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DRUG DEVELOPMENT - TUBERCULOSIS: IDENTIFICATION OF NEW MOLECULAR TARGETS

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Tuberculosis, a Health Problem Worldwide

Tuberculosis has been and continues to be the leading cause of deaths world wide from a single infectious agent. About one third of the human population is infected with *Mycobacterium tuberculosis*. According to World Health Organization estimate, between two and three million deaths occur each year due to this disease. Some eight to ten million new cases are reported each year and currently there are about 1,700 million total number of infected individuals (Blanchard, 1996). Tuberculosis has remained a severe problem in the developing countries. Since the outbreak of AIDS (acquired immune deficiency syndrome), it has resurfaced in the west. The spread of the AIDS virus in recent years is a contributing factor for the resurgence of the disease since tuberculosis is one of the most opportunistic infections in AIDS patients. This high degree of susceptibility to the tuberculosis is due to the immunosuppression resulting from infection with HIV (Bloom and Murray, 1992). Increases in the homeless population and declining health care structures and national surveillance have also contributed to the rise of tuberculosis (Snider and Roper, 1992).

Problem of Drug Resistance

Tuberculosis was considered to be a minor and decreasing problem in industrialized countries after the effective introduction of chemotherapy during the second half of this century. However, the disease remained a severe problem in developing countries. Recently, greater concern has been expressed world wide regarding the outbreaks of the disease due to an alarming increase in transmission of the multiple drug-resistant (MDR) tubercle bacilli (Perrone, 1993). Though the treatment of tuberculosis with multi-drug therapy witnessed a dramatic improvement in the control of the disease, the prolonged course of therapy (at least 6 months) and poor compliance, have resulted in the isolation of multiple drug resistant isolates of *M. tuberculosis*. This rise in multidrug-resistant tuberculosis infections worldwide has sounded a global concern to find effective ways to combat the disease.

The magnitude of this problem has prompted the researchers worldwide to develop better diagnostic and therapeutic agents to combat tuberculosis. Many scientific

approaches and public health efforts have been directed at diagnosis, treatment, and control of this disease. Identification of and further strategic manipulation of suitable molecular targets for new drugs to combat mycobacterial infections is an essential step to develop new antitubercular drug. An improved understanding of the molecular targets of antituberculosis drug and the biochemical mechanisms of drug resistance would facilitate the design of rapid diagnostic tests for detecting drug-resistant tubercle bacilli and the development of more effective therapeutics for treating multi-drug resistant tuberculosis (MDRTB). To combat disease with a drug, one need to have an ideal target, usually a cell component, which when acted upon by drug would lead to cell death or arrest in cell growth.

Known Drugs for Tuberculosis

An impressive number of antimycobacterials are used to combat mycobacterial infections. Some of the drugs such as isoniazid (INH), rifampicin etc have attained the status of front line drugs due to their effectiveness, dose response, less toxicity, lower side effects etc. The strains resistant to these first-line antituberculosis drug are mainly responsible for MDRTB outbreaks. Although the molecular events that cause drug resistance in *M. tuberculosis* have not yet been well defined in all cases, studies have been initiated to underly the mechanisms of resistances to these first-line antituberculosis drugs. Here, we have summarized the salient features of these drugs with respect to their mode of action, target molecule and resistant locus (**Table 1**). INH resistant mutants of *M. tuberculosis* frequently showed catalase negative phenotype which could be attributed to a deletion (Zhang *et al*., 1992) or some point mutations in *kat*G gene (Heym *et al*. 1995). A point worth noting here is that molecular basis of INH resistance has been delineated in great detail in early 70's at Indian Institute of Science, India. Extensive biochemical and some classical genetic studies provided considerable information on mode of action and molecular basis of INH resistance (Gayathri Devi *et al*., 1975; Gopinathan, 1981). These authors demonstrated that concomitant loss of calalase and peroxidase activity in INH resistance strains of *M. smegmatis* and *M. tuberculosis*. Recently, resistance to rifampicin was found to be determined by mutations causing change in the *rpo*B gene coding β-subunit of RNA polymerase (Williams *et al*., 1994). It was reported earlier that mycobacterial DNA-dependent RNA polymerase was 1,000 times more sensitive to rifampicin than the *E. coli* RNA polymerase (Harshey and Ramakrishnan, 1976). Other drugs available include ethambutol, pyrazinamide and streptomycin. Streptomycin, an aminoglycoside antibiotic inhibits protein synthesis (Shaila *et al*., 1973), causing misreading of codons during translation. Streptomycin-resistant mutations that affect the ribosomal protein S12 are thought to inhibit the ability of the drug to bind to the ribosome (Funatsu and Wittmann, 1972; Liu *et al*. 1989). The highly conserved *rpsL* gene, which encodes the S12 ribosomal protein, is known to be the primary site for streptomycin-resistant mutations in *M. tuberculosis* (Nair *et al*. 1993). Ethambutol and D-cycloserine are cell wall synthesis inhibitors. Ethambutol was able to significantly increase the bactericidal activity of other drugs by improving their entry (Rastogi *et al*. 1990 & 1991). D-cycloserine has not been very successful because it is a broad spectrum compound, having high toxic effects (Rastogi and David, 1993). The single amidase enzyme with both pyrazinamidase (PZase) and nicotinamidase activities (Konno *et al*., 1967) in *M. tuberculosis* is the target for pyrazinamide. PZase in *M. tuberculosis* converts PZA to bactericidal pyrazionic acid (POA) inside the bacterial cell (Bonicke and Lisboa, 1959;

Scorpio and Zhang, 1996). The mutations in *pnc*A gene encoding the amidase enzyme with both PZase and nicotinamidase activity confer resistance to pyrazinamide in *M. tuberculosis* complex organisms (Scorpio and Zhang, 1996; Sreevatsan *et al.*, 1997). Pyrazinamide has been found to act also on host's bactericidal system (Rastogi *et al.* 1988; Crowle, 1989), so its use as chemotherapeutic agent seems to be restricted.

Table 1
Well known antimycobacterial agents

Drug/ Antibiotic	Cellular function affected	Target molecules	Molecular basis of resistance
Rifampicin	RNA synthesis	RNA polymerase β-subunit	Point mutations in *rpoB* prevent drug binding
Isoniazid	Synthesis of mycolic acid components in cell wall -	NAD reductase (InhA) Catalase-peroxidase	Point mutations in *inh*A (A94S) Point mutations in *katG* gene
Streptomycin	Protein synthesis	S12 ribosomal protein	Mutations in *rpsL* gene
Ethambutol	Cell wall synthesis	-	-
Pyrazinamide	Change in pH	Amidase	Mutations in *pnc*A gene
Norfloxacin	DNA synthesis RNA synthesis	DNA gyrase-DNA complex	Single mutations in gyr A or gyrB
Novobiocin[a]	DNA synthesis RNA synthesis	DNA gyrase B subunit	Single mutation in gyrB affecting ATPase activity

[a] Not in the clinical trial only in laboratory experiments.

Criteria for an Efficient Drug Target

What could be an ideal target for drug discovery? (i) The protein or enzyme exhibiting important function essential for cell survival. (ii) It would be advantageous if the target enzyme is present only in bacteria and not in eukaryotes. (iii) On the other hand, if present ubiquitously the target molecule in bacteria should have characteristics distinct from the eukaryotic counterparts. (iv) It would also be desirable if the properties of the target enzyme in the pathogenic bacteria are different from those of the other bacteria.

Our Approach - DNA Topoisomerases as Drug Targets

From the above discussion, it is clear that there is immediate need to identify new molecular targets for drug discovery research. In this section, we describe our initial efforts in this direction. We have chosen DNA topoisomerases of mycobacteria as molecular target. A brief overview of these essential enzymes is provided here as a prelude to our investigation of these enzymes from mycobacteria.

DNA topoisomerases are essential for cell survival. They are found ubiquitously and play a vital role in variety of cellular processes by maintaining the superhelical density of DNA (Wang, 1985). Both prokaryotes and eukaryotes have been shown to possess multiple topoisomerases, possibly evolved to provide division of labour and in certain cases as backup strategies to take care of important cellular functions (Madhusudan and Nagaraja, 1996). As a consequence they influence various biological processes involving DNA, such as replication, transcription, recombination and chromosome dynamics. There are two distinct classes of topoisomerases (**Table 2**). Type I group consists of prokaryotic and eukaryotic topoisomerase I and topoisomerase III enzymes which effect topological changes in DNA by transiently cleaving one DNA strand at a time to allow the passage of another strand resulting in change in linking number of DNA by steps of one (Maxwell and Gellert, 1986). Eukaryotic topoisomerase II, bacterial DNA gyrase and topoisomerase IV belong to the type II family. The enzymes of the latter group require ATP for catalysis and introduce transient double standard breaks, changing the linking number in steps of two. Type I topoisomerases in conjunction with type II enzymes maintain the superhelical density *in vivo*. Both type I and type II enzymes have been found to participate in nearly all cellular transactions involving DNA. The prokaryotic type I enzymes differ substantially from their eukaryotic counterparts in DNA cleavage characteristics. In the cleavage step, the prokaryote enzyme-DNA covalent intermediate is formed between a tyrosine residue of the enzyme and the 5'-phosphate at the DNA break site while in the case of eukaryote counterpart, it is the 3'-phosphate end involved in the covalent intermediate formation (Roca, 1995). Thus, by having a distinct feature to that of eukaryotic enzymes, the prokaryote topoisomerase I (especially the one from mycobacteria) meets one of the requirements of a target molecule for the development of antimycobacterial drugs.

DNA gyrase, a type II topoisomerase is perhaps an ideal target. Two of its important characteristics *viz.*, an enzyme available only in bacteria and is the only enzyme that introduces negative supercoils into DNA justifies the point. DNA gyrase is a type II topoisomerase consisting of two subunits GyrA and GyrB that form an active complex A_2B_2, constituting the active form of the enzyme. DNA gyrase has been isolated from many bacterial species and well characterised from *E. coli*. It was isolated in 1976 (Gellert, *et al.* 1976a) and serves as a prototype enzyme (Cozzarelli, 1980; Reece and Maxwell, 1991). The basic properties of the two subunits GyrA and GyrB, of *E. coli* gyrase are summarised in **Table 3**. The mechanism of different steps involved in the supercoiling activity has been well studied and involves the following steps (1) wrapping

of a segment of DNA (≈130 bp) around the enzyme in a positive superhelical sense. (2) cleavage of the wrapped DNA in both strands with the formation of covalent bonds between the newly formed 5'phosphates and Tyr122 of the A subunits. (3) passage of other segment of DNA through this double-strand break, and (4) resealing of the broken DNA leading to change in the linking number in steps of two. Catalytic supercoiling is coupled energetically to ATP hydrolysis. Apart from negative supercoiling reaction, the enzyme catalyses relaxation of the DNA, catenation-decatenation and knotting-unknotting reactions. A wealth of information is available on structure-function and mechanism of action of the DNA gyrase from *E. coli*.

Table 2
DNA topoisomerase families.*

Family	Characteristics	Enzyme	Gene	Organism
Type 1-5'	Cleave single DNA strands, forming 5'-phosphotyrosine covalent intermediate	Bacterial DNA topoisomeraseI	TOPA	*Escherichia coli*
		Bacterial DNA topoisomerase III	TOPB	*Escherichia coli*
		Eukaryotic DNA topoisomerase III	TOP3	*Saccharomyces cerevisiae*
		Reverse gyrase		*Sulphobus acidocaldarius*
Type 1-3'	Bind duplex DNA and cleave one of the strands, forming 3'-phosphotyrosine covalent intermediate	Eukaryotic DNA topoisomerase	TOP1	*Saccharomyces cerevisiae*
		DNA topoisomerase V	TOP1	*Drosophila melanogaster*
		Vaccinia virus topoisomerase	TOP1	*Homo sapiens*
		Variola virus topoisomerase		*Methanopyrus kandleri*
		Shope fibroma virus topoisomerase		
Type II	Cleave both strands of duplex DNA, forming a pair of 5'-phosphotyrosine covalent intermediates. Functions as dyadic enzymes and are ATP dependent	Bacterial DNA gyrase	GyrA + GyrB	*Escherichia coli*
		Bacterial DNA topoisomerase IV	ParC + ParE	*Escherichia coli*
		Eukaryotic DNA topoisomerase II	TOP2	*Saccharomyces cerevisiae*
			TOP2	*Drosophila melanogaster*
		Bacteriophage T4 topoisomerase	TOP2 α	*Homo sapiens*
		African swine fever virus topoisomerase	TOP2 β	*Homo sapiens*
			gp39 + gp60 + gp52	

*Reproduced from Roca, 1995.

Table 3
Properties of *Escherichia coli* DNA gyrase[a]

Properties	GyrA subunit	GyrB subunit
Gene	gyrA (2625 bp, formerly *nal*A)	gyrB (2412 bp, formerly *cou*)
MW (Da)	96 756 (874 amino acids)	89 762 (803 amino acids)
Major role	Breakage and reunion of DNA	ATPase activity
Drug interaction	Quinolones, clerocidin?, Ccdb	Quinolones?, coumarins, cyclothialidines, microcin B17

[a] Reproduced from Maxwell, 1997.

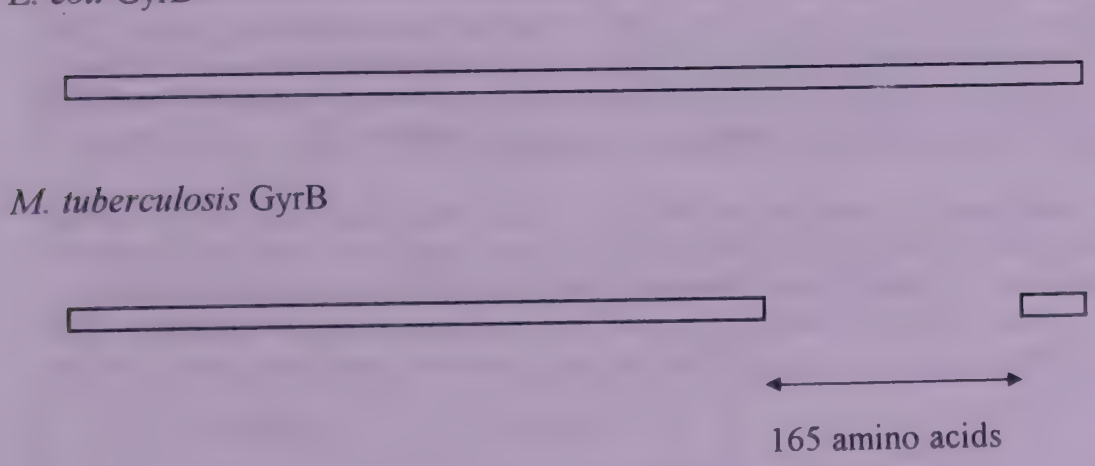

Figure 1. Schematic representation of sequence comparison between *M. tuberculosis* GyrB and *E. coli* GyrB

Quinolones and coumarins are the known inhibitors of the DNA gyrase. Nalidixic acid and oxolinic acid are the prototype quinolones while novobiocin and coumermycin A1 are the best known coumarin inhibitors of the enzyme (Maxwell and Gellert, 1986; Gellert *et al.*, 1976b). Novobiocin and coumermycin A1 are isolated from *Streptomyces*. The drugs have been shown to be the inhibitors of the ATPase activity of GyrB subunit. The enzymic analysis of 43 kDa amino-terminal domain of GyrB shows coumarin-sensitive ATPase activity, which localizes the coumarin drug-binding site to this part of the protein (**Figure 1**). Analysis of coumarin-resistant bacterial strains has identified point mutations to coumarin resistance that map to the 24-kDa amino-terminal subdomain of

gyrB (Maxwell, 1993). Quinolones are synthetic compounds rather than being natural products. Quinolones bind to Gyrase-DNA complex, thereby arresting the covalent intermediate of supercoiling reaction. They serve as excellent antibacterial agents (Maxwell, 1992) and fluoroquinolones have become successful as drugs (Domagala, 1994; von Rosentiel and Adam, 1994). Following the discovery of DNA gyrase, it was shown that the the supercoiling activity of gyrase could be inhibited by oxolinic acid, and that gyrase extracted from a quinolone-resistant *E. coli* strain was resistant to oxolinic acid. Many quinolone resistance mutations have been mapped to the gyrase genes, principally gyrA and also to gyrB (Maxwell, 1992). The quinolones inhibit the DNA gyrase activity by trapping the otherwise transient enzyme-DNA covalent intermediate of the topoisomerase reaction (Snyder and Drlica, 1978; Liu, 1989). The major problem in the usage of quinolones as drugs is the development of resistant strains at high rate (Wolfson, *et al.* 1989). Coumarins, on the other hand, have been confined to the laboratory experiments, due to their high toxicity.

There are several inhibitors of gyrase that fall outside the coumarin and quinolone family. Microcin B17 (MccB17), a glycine-rich peptide, is known to inhibit the growth of enterobacteria (Yorgey *et al*, 1994).. The subunit B of gyrase has been found to be the target molecule for this peptide. MccB17 resistance mutations have been mapped to gyrB genes in *E. coli* (Vizan, *et al.*, 1991). Cyclothialidine is a cyclic peptide isolated from *Streptomyces* (Goetschi, *et al.*, 1993) which inhibits the supercoiling and ATPase activity of DNA gyrase (Nakada, N. *et al.*, 1994). CcdB, the killer protein of F plasmid, exhibits antibacterial activity by a mechanism involving DNA gyrase. Mutations confering resistance to CcdB map to gyrA (Bernard and Couturier, 1992; Miki, *et al.*, 1992).

The background information on DNA gyrase and other topoisomerases described above form the basis for the study of these enzymes from mycobacteria in our laboratory. The primary objective of our approach to study molecular biology of mycobacteria is to identify and exploit suitable molecular targets for candidate drug development. This approach also allows to address the problem of drug resistance and basic biology of the system.

Table 4.
Effect of quinolones and coumarins on DNA gyrase supercoiling activity

Compounds	Concentrations	% Activity *M. smegmatis*	*E. coli.*
Control	--	100	100
Novobiocin	2 µg/ml	<1	<1
Coumermycin	2 µg/ml	<1	<1
Ciprofloxacin	20 µg/ml	20	<1
Norfloxacin	20 µg/ml	30	<1
Ofloxacin	20 µg/ml	20	<1
Oxolinic acid	200 µg/ml	50	<1
Nalidixic acid	200 µg/ml	15	40

Table 5
Inhibitors of DNA Topoisomerases

Class of drugs	Examples	Topoisomerases Inhibited
Coumarins	Novobiocin Coumermycin A1 Clorobiocin	Bacterial Gyrase (B subunit) Eukaryotic Topoisomerase II Reverse Gyrase Vaccinia Type I Topoisomerase
Quinolones	Nalidixic Acid Oxolinic Acid Norfloxacin	Bacterial Gyrase (A subunit) Phage T4 topoisomerase
Acridines	m-AMSA	Eukaryotic Topoisomerase I phage T4 topoisomerase
Anthracyclines	5-iminodaunorubicin	Eukaryotic Topoisomerase II
Ellipticines	2-me-9-OH-E+	Eukaryotic Topoisomerase II
Elliptophyllotoxins	VP-16 VM-26	Eukaryotic Topoisomerase II
Alkaloids	Camptothecin	Eukaryotic Topoisomerase I

Table 6
Effect of topoisomerase I inhibitors

Compounds	Concentration	% Activity* *Smegmatis*	*E. coli.*
Control	----	100	100
Camptothecin	50 µM 200 µM 500 µM	100 70 10	100 75 <5
Oxolinic acid	15 µg/ml 150 µg/ml	100 95	100 10
Norfloxacin	15 µg/ml 150 µg/ml	100 60	90 20
Novobiocin	150 µg/ml	100	100

*The enzyme activity in control (no drug) is taken as 100 % after scanning the gel. ND: Not determined.

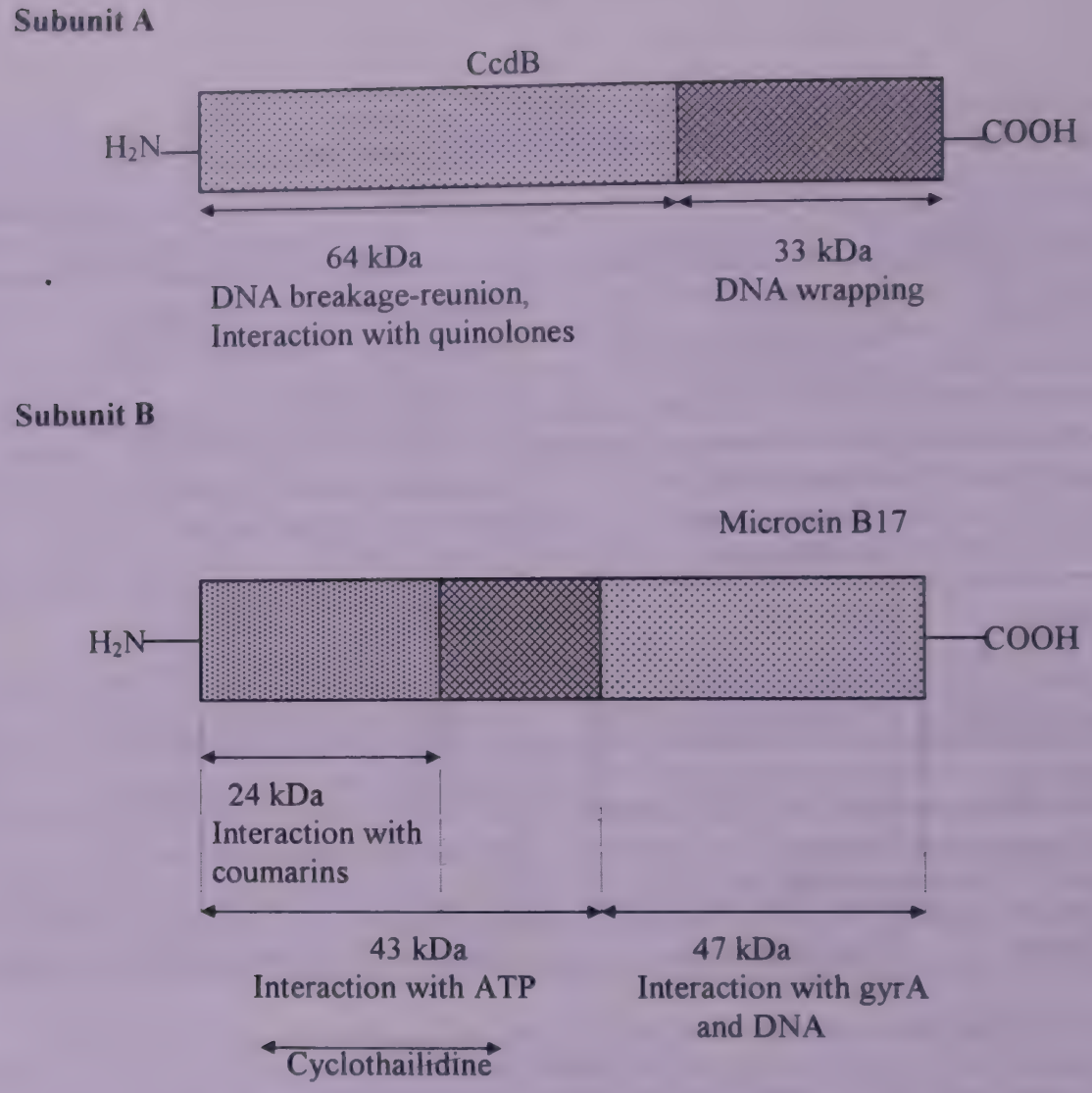

Figure 2. Domain structure of *Escherichia Coli* DNA gyrase. Comparison of drug binding sites.

As explained above, DNA gyrase with its unique characteristics and structural features could be a suitable molecular target for anti-mycobacterial drugs. DNA gyrase supercoiling activity was detected in *M. smegmatis* in the early 80's (Nagaraja & Gopinathan, 1981). In our lab, we have recently cloned gyrA and gyrB from *M. tuberculosis* and *M. smegtmatis* (Madhusudan *et al*, 1994, 1994, 1995). The genes have been sequenced and compared with other gyr sequences available. It is interesting to mention here that a stretch of 165 amino acids as found in the prototype *E. coli* gyrB is absent in gyrB from mycobacteria (**Figure 2**). The genes have been cloned into expression vetors to overproduce the enzyme in large quantities. The DNA gyrase from *M. smegmatis* has been purified as a holoenzyme. Screening of a number of new preparations of natural and synthetic origin for the inhibition of the enzyme is underway. **Figure 3** shows the typical pattern of *M. smegmatis* gyrase supercoiling inhibitory activity of certain known quinolones and coumarins. **Table 4** shows the comparative account of the inhibitory activity of known compounds against gyrase from *M. smegmatis* and the prototype enzyme from *E. coli*. Distinct properties of mycobacterial enzyme would form the basis to use it as a new molecular target. In a parallel study, we have purified topoisomerase I from *M. smegmatis* to apparent homogeneity and characterized the enzyme

biochemically (Bhaduri *et al.* 1994, 1998a, 1998b). The enzyme seems to be the largest single subunit prokaryotic type I topoisomerase known till date. We have shown that the mycobacterial enzyme is distinct in characteristics from prototype *E. coli* enzyme. The mycobacterial enzyme shows unusual thermostability and broad pH optima. Unlike the *E. coli* counterpart, mycobacterial enzyme does not contain bound zinc atoms and cysteine residues do not play any role in the DNA relaxation activity of the enzyme. Distinct properties of mycobacterial enzyme would form the basis to use it as a new molecular target. A large number of compounds have been identified as specific inhibitors of topoisomerases (**Table 5**) from different organisms. However, no compound has been shown to inhibit prokaryotic topoisomerase I at physiologically relevant concentrations. **Table 6** shows the effect of different topoisomerase specific drugs on the topoisomerase I activity from *M. smegmatis* and *E. coli*. The compounds tested were camptothecin, oxolinic acid, norfloxacin and novobiocin. Camptothecin is a plant alkaloid which inhibits eukaryotic topoisomerase I within the concentration range of 10-50 µg/ml (Hsaing *et al.* 1985). Only at a very high concentrations of camptothecin (500 µM) and norfloxacin (150 µg/ml), relaxation activity was partially inhibited, while oxolinic acid had no significant effect on enzyme activity. Novobiocin had no inhibitory effect on enzymes from either sources.

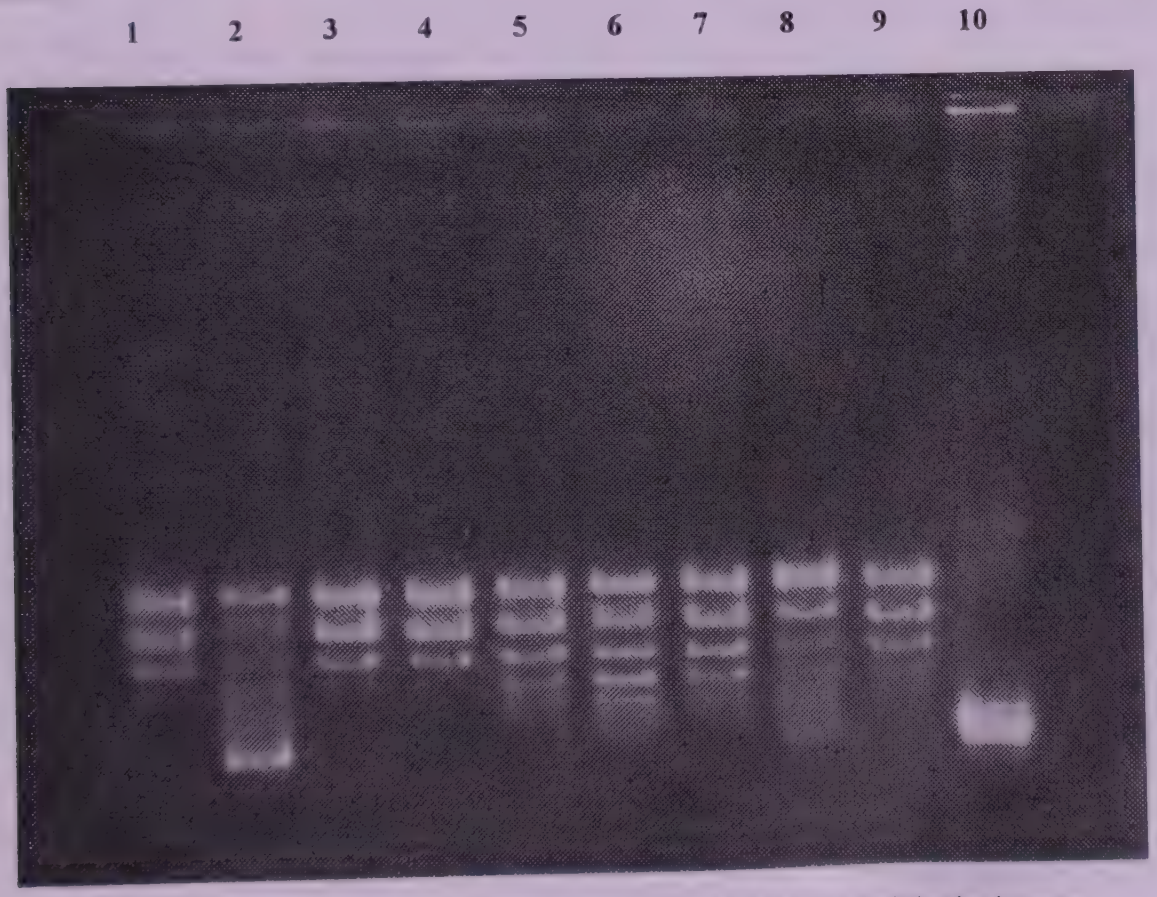

Figure 3. The *M. smegmatis* DNA Gyrase supercoiling inhibitory activity by known quinolines and coumarins. Lanes 1, pUC18 DNA (relaxed), 2, supercoiling activity, Lanes 3-9, assay mixture + inhibitors 3, novobiocin (2 µg/ml); 4, coumermycin A1 (2 µg/ml), 5, ciprofloxacin (20 µg/ml), 6, norfloxacin (20 µg/ml), 7, ofloxacin (20 µg/ml), 8, oxolinic acid (200 µg/ml), 9, nalidixic acid (200 µg/ml); 10, supcoiled pUC18 DNA alone

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TUBERCULOSIS VACCINE STRATEGY

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This presentation will discuss first the current strategy for vaccination against tuberculosis, including its rationale, its practice and its implications, and then turn to future strategies, based upon old or new vaccines.

Current Strategy

BCG is our only current vaccine against tuberculosis. There is a long history of controversy about BCG, attributable mainly to variation in efficacy observed in different studies and populations. The largest formal trial, carried out in the Chinglepet area of South India, revealed little if any efficacy against adult forms of tuberculosis (1).

The current strategy for BCG use, as recommended by the WHO, is for only one dose, at birth. This strategy is emphasized for two reasons: (i) all studies to date have shown consistent protection by BCG against the severe childhood forms of tuberculosis, in particular meningitis(2); and (ii) there is no evidence that repeated BCG, or booster doses, increases protection against tuberculosis. Some countries still follow other BCG strategies, such as primary vaccination of school children (eg the UK, Denmark) and repeat vaccination (eg several Eastern European nations). BCG is now routine in most countries of the world, and is given to approximately 100 million children per year.

The impact of current BCG programmes against tuberculosis is difficult to assess, for several reasons: (i) because several factors relevant to tuberculosis (eg socio-economic conditions, tuberculosis case finding and treatment activities, and now HIV) have changed in most populations in recent decades, along with the introduction and increasing use of BCG; (ii) good long term data are available on very few populations; and (iii) the major tuberculosis burden is in old people, most of whom were born too early to have been included in BCG vaccination programmes. For such reasons it is difficult to attribute any changes in gross tuberculosis patterns, as observed in various countries, to BCG, except for declines in certain vaccinated cohorts shortly after introduction of BCG.

A major issue affecting BCG's strategy and impact is the duration of protection by the vaccine. This is crucially important, given that although BCG is generally administered to children, the major burden of tuberculosis occurs in adults, and in particular older adults. There are few studies which allow any evaluation of protection beyond ten years after vaccination, and the available data show no consistent pattern in protection over time[3].

Long-term follow-up of trial populations is difficult, and in any case most of the trials have been too small to allow long term follow up. Observational studies of long-term protection are difficult, in particular because of the difficulty of documenting vaccinations carried out long ago. The validity of BCG scars as a record of past vaccination deteriorates with time and age.

Although WHO does not now recommend repeat vaccination, it must be noted that there are almost no data on which to base this decision. The only formal evaluation of a second BCG was carried out in a trial in Malawi, which found, that repeating a BCG vaccination increased the protection imparted by the initial dose against leprosy, but it had no observable effect on (pulmonary) tuberculosis incidence (4). It must be emphsized that this study was carried out in a population where the initial BCG vaccination appeared to provide appreciable protection (50-70%) against leprosy, but none at all against tuberculosis. Thus this trial might be interpreted as evidence that a repeat BCG can provide additional protection but only in populations where a primary vaccination provides some protection. This issue of the duration of BCG's protection, and of the potential utility of BCG boosters, is one of the very important issues for tuberculosis vaccine research today.

Our interpretation of BCG's behaviour is inevitably linked to our interpretation of the natural history of tuberculosis. There is good evidence that clinical tuberculosis can arise either soon (within months or a few years) after an initial infection, or it may arise many years later, either through reactivation of an "old" dormant infection or else by reinfection. Each of these mechanisms has implications for underlying immunological processes. Unfortunately, there is little consensus on the extent of disease of each sort which arises in different populations. It has been proposed that one explanation for the apparent poor protection by BCG in Chinglepet might have been because much of the disease was attributable to reinfection in individuals who had already experienced a primary infection, and thus who had already acquired as "immunity" as BCG is able to impart. This explanation is related to another hypothesis for the failure of BCG in several (especially) tropical populations - that these populations are exposed to many environmental mycobacteria, which sensitize individuals, and in effect provide as much protection as can be given by BCG. Resolution of these questions, which relate the pathogenesis of tuberculosis to protection provided by BCG and to natural exposure to environmental mycobacteria is another crucially important topic for research today.

All such studies are made difficult by the absence of any correlate of natural or vaccine-derived protective immunity against tuberculosis. The only immunological measure relevant to tubercle experience which has been studied extensively is tuberculin sensitivity. In addition to the difficulty of interpreting tuberculin reactivity in populations exposed to environmental mycobacteria, the relationship between tuberculosis and prior tuberculin sensitivity in many populations is non-linear (U-shaped), with the lowest risk associated with low-moderate levels of tuberculin sensitivity (5). Whether the low risk of tuberculosis among individuals with low-moderate levels of tuberculin sensitivity reflects their prior exposure to environmental mycobacteria or whether it reflects that these people

have been infected with *M tuberculosis*, but have effectively dealt with that initial exposure, is not known. This is a subject which needs innovative work, employing some of the newer tools for evaluating cellular immunological responses. An example of the sort of work which should be encouraged is the study which compared BCG-induced enhancement of macrophage killing of *M.microti* between populations in the Chinglepet area with populations in the UK (6).

Though BCG is primarily a tuberculosis vaccine, it is also important to note that it is equally if not more effective against leprosy, and that the wide use of BCG is probably having a major effect of leprosy incidence worldwide. For this reason decisions of its use should consider implications for leprosy as well as for tuberculosis.

Future Strategy

The major strategy issue relating to the future use of BCG concern (i) resolution of the old problem of the reasons for different efficacy between populations; (ii) the utility of boosters, (iii) the identification of correlates which will indicate the effectiveness of BCG among individuals in different populations, (iv) measurement of BCG's impact against different forms of childhood tuberculosis and leprosy, as well as against pulmonary tuberculosis, and (v) the implications of increasing HIV prevalence for BCG.

The current revival in tuberculosis research, and interest in a new tuberculosis vaccine, is attributable largely to the immense burden of adult pulmonary tuberculosis - it is this form of the disease which has made tuberculosis a "global emergency". The problem of developing (and evaluating) a (new) vaccine against this form of tuberculosis will be difficult, for immunological as well as logistic reasons.

Immunologically, we need a vaccine which does that which BCG fails to do (ie protect consistently against adult tuberculosis) - for reasons which we do not understand. Among the issues are the problems of imparting long-term protection in individuals who have been sensitized, sometimes repeatedly, to a variety of different mycobacteria, perhaps including *M tuberculosis* itself. If, indeed, much adult tuberculosis occurs in individuals who have long before met (and coped at least temporarily with) the tubercle bacillus, how can we manipulate the immune system to do that which exposure to the homologous antigens appears to have failed to do - or at least only did for a limited period of time?

The logistic problem arises when considering the evaluation of a new vaccine. Ideally we would like a vaccine which could be given to children, along with all the other vaccines, and which will protect them for life. But this poses almost inseparable difficulties for tuberculosis - as it is difficult to conceive of a trial starting in the next few years in children, which will follow them for the length of time needed to see if they are protected against adult forms of tuberculosis decades hence. For this reason, we will probably have to try out the vaccine in adults - which means giving the vaccine to individuals who already have a complicated cumulative experience of mycobacterial

exposure, often including various environmental mycobacteria, BCG, and perhaps *M.tuberculosis* itself. Not only is this a messy background against which to evaluate a vaccine, but we will still face the problem of evaluating duration of protection, and whether booster doses are required - which has considerable implications for the sample size of any trial (if, for example we wish to randomize the vaccinated group to receive a booster or not, five or ten years after the primary vaccination.

The task ahead in tuberculosis vaccine research is not a simple one. On the other hand, we have the advantages of a rapid increase in understanding of cellular immunity and the recent availability of cytokine assays which can be applied on an epidemiological scale. In addition, we have the advantage of the recent knowledge of the entire *M tuberculosis* genome, which provides access to the entire antigenic and epitopic repertoire of the tubercle bacillus. And we have the experience of BCG vaccines - which, despite its complexity, does show us that some tuberculosis vaccines can provide protection, under certain circumstances. We can use this experience to help understand why they worked where they did, and how we can ensure that this occurs consistently, everywhere.

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AN IMMUNOGENIC 28 KDA DNA BINDING PROTEIN (HLP_{MT}) OF MYCOBACTERIUM TUBERCULOSIS.

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Introduction

Several laboratories are currently engaged in isolation of antigens of *M.tuberculosis* which are targets of human immune response. This has become necessary as none of the reported antigens have led to the successful designing of a novel immuno-therapeutic or immuno-diagnostic reagents for tuberculosis. This failure may be partly due to the fact that antigenic components / epitopes recognized by immunocompetent cells during natural infection or disease in humans may significantly differ from those identified in experimental tuberculosis (1). In this study a systematic approach was undertaken to identify and characterize targets of human immune response in non - tuberculous healthy contacts. Using the Tcell blot assay and subtractive immuno-blotting techniques, a novel histone-like protein of mycobacteria was identified. This protein has homology to prokaryotic HU proteins and H1 histones from several different eukaryotic species.

MATERIALS AND METHODS

Subjects: Samples were collected from healthy residents of endemic areas / who had close contact with patients . Prior to inclusion in the study, the contacts, were screened for clinical signs and symptoms of tuberculosis. Twenty-six of them were tuberculin reactive showing > 5mm diameter of induration following an intradermal injection of 1 T.U. of tuberculin (BCG vaccine Laboratory, Madras, India) and 18 individuals showing less than 5mm induration were classified as non-reactors.

T-cell Blot Assay: SDS-PAGE - fractionated nitrocellulose-bound antigens of *M.tuberculosis* (H37Rv, Tuberculosis Research Center, Madras, India) for the lymphoproliferative assay were prepared as described by Abou-Zied et al. (2).

Statistical Analysis: The median test (3) was used for testing the significant difference in the median values between the two groups.

Immunoblot Assay: The mycobacterial proteins were resolved on a 5-20% gradient SDS-polyacrylamide gel and electroblotted on to nitrocellulose. The transblots were exposed to human sera (1:100) and the binding of human antibodies was visualized using peroxidase conjugated to anti-human IgG (DAKO, Denmark) and the appropriate substrate.

Subtractive immunoblotting : It was carried out on identical transblots initially treated with a battery of murine monoclonal antibodies. The treated blots were probed with human sera. The binding of human antibodies was visualized as before.

Partial Purification Of The 30 kDa Group Of the mycobacterial protein (s) was carried out by electroelution from excised gels (BIOTRAP, Schleicher & Schuell). The extracted protein (s) was electrophoresed on a 12% SDS-PAGE gel and electroblotted. The immunoreactivity of the eluted proteins was confirmed with pooled human sera. The nitrocellulose-bound antigens was further processed and used for generation of murine polyclonal sera (7). The amino acid sequence analysis of the eluted proteins was carried out by the Rockefeller University Protein Sequencing Facility, New York.

PCR Analysis and Sequencing: Polymerase chain reaction (PCR) was performed with Taq DNA polymerase according to the manufacturer's instructions (Boehringer Mannheim, Germany), using total genomic DNA of *M .tuberculosis* H37Rv and synthetic oligonucleotides HLPMNdeI (5'- GGA GGG TTC ATA TGA ACA AAG CAG-3') and HLPMSalI (5'- GTA TCC GTG TGT CGA CAC CTA TTT G-3'). The PCR-amplified product was cloned in pGEMT vector (Promega, Madison, USA) and sequenced using universal M13 and gene specific oligonulcleotide primers by the di-deoxy chain termination method (4) using Sequenase version 2.0 kit (USB, Amersham, USA.).

Cloning and Expression: The PCR-amplified product was purified and cloned in pT7-7 expression vector (9). This enabled the expression of the complete hlp_M gene from the ATG initiation codon. DNA from the recombinants was transformed in *E.coli* K-38 cells containing pGP1-2 and the cells were heat induced at 44°C to check for expression. SDS-PAGE and immunoblotting of the *E.coli* cell lysates was performed as described using the anti-30 kDa monospecific sera.

South-Western Blotting: Transblots of resolved mycobacterial proteins were blocked with PBS containing 5% bovine serum albumin .^{32}P- labeled DNA probe from λ phage (New England Biolabs, Inc. Beverly, MA, USA), pGEM3Z f (+) plasmid (Promega, Madison, USA) and total genomic DNA of M.tuberculosis (Tuberculosis Research Materials, NIAID, NIH) were prepared by using the random priming kit (New England Biolabs Inc. Beverly, MA, USA). The washed blots were probed for 2hr at room temperature with labeled DNA. The unbound label was removed by washing serially with PBS-Tween and PBS. The blots were air dried and subjected to autoradiography (5).

Results

Identification of antigens of *M.tuberculosis* associated With Human immune Response: Two immunological assays were used to identify unique antigens of *M.tuberculosis* associated with human immune response namely T-cell blot and immunoblot assays.

T-cell blot assay: PBMC isolated from 44 healthy individuals (26 tuberculin reactors and 18 non-reactors) were co-cultured with nitrocellulose-bound fractionated antigens of *M.tuberculosis* as described in methods. Comparing the stimulation indices obtained in tuberculin reactors with those determined for non-reactors, showed there were significant differences between the two groups in 18 of the 28 fractions. ($p< 0.01$ to < 0.0003) . The stimulation indices of tuberculin reactors and non-reactors responding to each of the 28 fractions has been depicted in Fig: 1. The threshold for considering a fraction to be immunogenic was established by: (a) by comparing the lymphoproliferative responses of the two groups to a nitrocellulose-bound mycobacterial fraction and (b) taking into consideration the fraction which gave a stimulation index of 6 or > in 60% of the individuals.

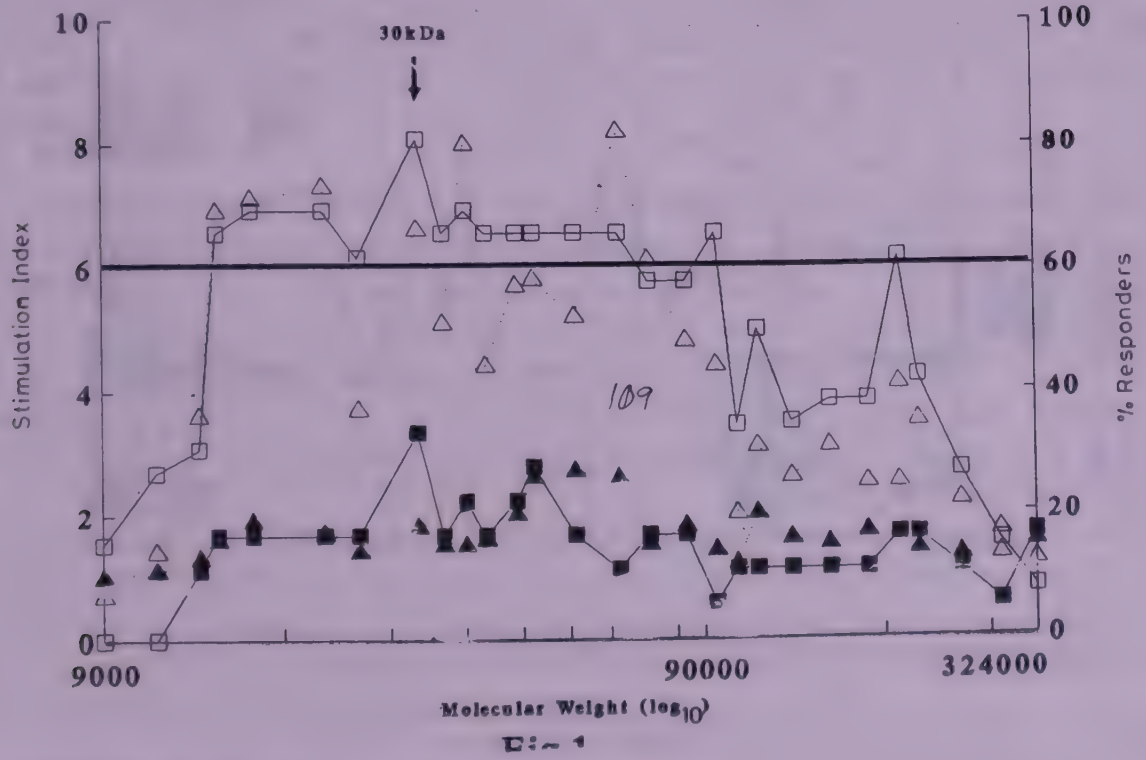

Fig 1: Proliferative responses of human peripheral blood mononuclear cells (PBMC) derived from healthy non-tuberculous contacts (Tuberculin Reactors) to the 28 fractionated *M.tuberculosis* antigens bound to nitrocellulose (T cell Blot Assay). The stimulation indices of the 26 reactors to each of the 28 fractions (9-324 kDa) has been depicted. The immunogenic fraction 21 is indicated. (→).

Fraction 21 (30 kDa) was considered to be the immunogenic fraction in the T cell blot assay among the tuberculin reactors included in the study.

Immunoblot assay And Subtractive Immunoblot Assay: The serum / plasma of the donors were screened for the presence of anti - *M.tuberculosis* antibodies by immunoblotting. In all samples antibodies reactive with a wide range of mycobacterial antigens ranging in molecular weight from 116 to less than 24 kDa were detected. (Fig: 2). The transblots initially exposed to a panel of murine monoclonal antibodies followed by exposure to individual sera / pools of sera. The results were identical in both cases. The residual reactivity seen in Fig: 2B. It appears that the murine monoclonal antibodies blocked / sterically interfered with the binding of human antibodies. over a 35 to 71 kDa range. However monoclonals specific for antigens of 16,17-19, and 30 kDa failed to inhibit the binding of human antibodies to these antigens. The subtractive immunoblot assay showed the prominence and residual reactivity of the sera with the 30 kDa antigen irrespective of the source of the sera, (Fig: 2B).

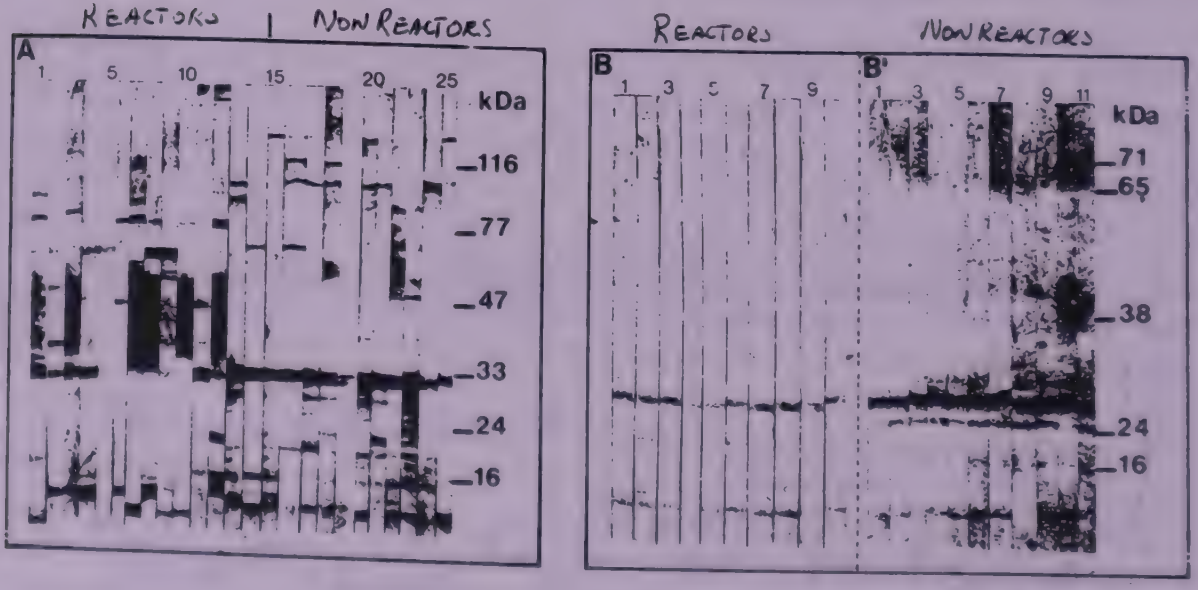

Fig 2:Representative immunoblots of *M.tuberculosis* sonic extract with sera / plasma (1:100 dilution) of healthy contacts. The position of the molecular weight markers in kDa are indicated on the right Tuberculin reactors (Lanes 1-10); tuberculin non - reactors (Lanes 11-20).

Fig2B: Representative immunoblot probed for the binding of human sera on transblots initially treated with murine monoclonal antibodies. Note the persistent immuno-reactivity with human sera in the 30 kDa region and less. The molecular weight markers are indicated on the right.

For further characterization the protein (s) associated with fraction 21 (30 kDa) were taken up.

Polyclonal Sera For The 30 kDa Mixture of Proteins: Transblots of *M.tuberculosis* sonicates and culture filtrate proteins were probed separately. A single band at a molecular weight corresponding to 30 kDa was seen. No band was detected with the corresponding culture filtrate antigens indicating that the antigen(s) is associated with the mycobacterial cell and is not secreted into the culture fluid , unlike proteins of the 85 ABC complex which are primarily secretory proteins.

Protein Sequencing: Four peptide sequences were obtained on digestion of the 30 kDa proteins with endoproteinase Lys-C (11). The 16-mer peptide sequence was determined to be - V K P T S V P A F R P G A Q F K.

Identification and Cloning of hlp_{Mt} gene of *M.tuberculosis*: The origin of the 16-amino acid peptide, was established with the mycobacterial database sequences maintained at the Sanger Center, Cambridge, U.K.. A 100% homology was obtained with the translated sequence of a region of the cosmid cY349. The nucleotide sequence of the cosmid cY349 was analyzed for an ORF that was in frame with the experimentally determined peptide sequence (VKPTSVPAFRPGAQFK) using DNASIS program (Pharmacia, Sweden). Such an ORF was obtained in the first frame which comprised 646 bp and mapped between nucleotide position 274 and 916 bp in cosmid cY349 . The ORF was predicted to encode a protein of 214 amino acids with a molecular weight of 22 kDa .

Computer-aided analysis of hlp_{Mt} gene and its gene product: Fickett analysis (DNASIS program) showed that the 646 bp hlp_{Mt} ORF was a protein-coding region and the gene has a 74% bias for G/C in the third position of the codon, similar to other mycobacterial genes. The HLP_{Mt} has characteristics typical of basic DNA binding proteins. However, unlike similar reported proteins of chlamydia (13) which are homologous to H1 histones, HLP_{Mt} shows sequence homology to both bacterial histone-like proteins (HU) as well as to eukaryotic H1 histones.

At the amino acid level a homology of 48.3% in a 89 amino acid overlap (1-90) was obtained with the HU protein of *Bacillus stearothermophilus*. The remaining 114 amino acid stretch (C-terminal) showed a 43.8% homology in a 112-amino acid overlap with late H1 histone of the sea urchin and a 45.2% homology in a 115-amino acid overlap with the AlgP protein of *Pseudomonas aeruginosa*..

Expression of the hlp_{Mt} Gene of *M.tuberculosis*: The coding potential of the predicted ORF was confirmed by its overexpression in *E.coli*. Oligonucleotides were designed from the 5'and 3'regions of the gene and restriction enzyme sites *NdeI* and *SalI* were incorporated into the primers to facilitate cloning of the complete ORF. The ORF was PCR-amplified from genomic DNA of *M.tuberculosis* H37Rv . The 675-bp amplified product was digested with NdeI and SalI and the complete 646 bp ORF was cloned in the expression vector pT7-7 (9) to yield construct pHLPM. The plasmid (pHLPM) was used to transform *E.coli* K-38 cells for expression. The *E.coli* cell lysates were

analyzed on a gradient SDS-PAGE gel. A 28 kDa band was visualized in the heat-induced culture lysates which was absent in the uninduced culture lysates.. A discrepancy between the calculated molecular mass (22 kDa) and that estimated from SDS-PAGE (28 kDa) was observed, which is most probably due to the highly basic nature of the protein. The electrophoresed *E.coli* lysates were electroblotted on to nitrocellulose membrane and probed with anti-30 kDa antibody. The antibody reacted exclusively with the expressed 28 kDa band which was absent in the uninduced and vector cell lysates. In the mycobacterial sonicate, a faint band at a molecular weight corresponding to 28 kDa and a prominent and at 30 kDa was obtained. This could possibly be due to the fact that the monospecific sera is either reactive with more than one protein or there are differences due to post translational modification of the protein in *M.tuberculosis* . The presence of six potential phosphorylation sites in the sequence of HLP_{Mt} was suggested following analysis by the GCG program .

South-Western analysis: In order to evaluate whether the HLP_{Mt} could bind DNA, total *E.coli* cell lysates were electrophoresed and electroblotted in the presence of 250mM NaCl and this binding was not detected either in the uninduced or in vector cell lysates. This indicates that the HLP_{Mt} binds to DNA in a non-specific manner nitrocellulose filters. Fig 5 shows that the DNA probes bound the HLP_{Mt} protein.

Discussion

The results presented in this study demonstrate that immunological assays can be used to identify and characterize new antigens of *M.tuberculosis* associated with human immune responses. The 30 kDa group of proteins were found to be immunodominant in the T-cell blot assay. Antigen(s) in a similar molecular weight range (24 - <33 kDa) were found to be immuno-reactive by the immunoblot assays. The 30 kDa group of proteins (fraction 21) induced vigorous lymphoproliferation in PBMCs of healthy tuberculin reactors while the response in non-reactors was limited.

The cloned HLP_{Mt} gene was confirmed by DNA sequencing and was found to be identical to the one determined by the Sanger Center (UK). The presence of one copy of the HLP_{Mt} gene in the mycobacterial genome was confirmed by Southern analysis. (data not shown). HLP_{Mt} is novel in that its N-terminal exhibits significant homology to the bacterial HU proteins particularly in those amino acid residues implicated in DNA interaction (16), while its C-terminal segment displays homology to eukaryotic H1 histones. There have been recent reports of mycobacterial proteins have properties similar to eukaryotic proteins such as serine / threonine kinase and tRNA. (6, 7).

The homology of HLP_{Mt} is to the C-terminal region of H1-histone, which is known to bind linker DNA in eukaryotes and to be involved in higher order folding of polynucleosomes (29). It has been proposed that binding of histone H1 to DNA stabilizes formation of α-helical structure in the C-terminal domain of histone H1,

Interestingly computer programs predicting protein secondary structure indicate that HLP_{Mt} could form long α-_helical structures within the tail (data not shown) that could interact with DNA. This is probably due to its high lysine and alanine content, which strongly favors α-helical structure. The HLP_{Mt} has seven tetrapeptide repeats (PAKK and KAAK) in the 118 to 200 amino acid stretch. These tetrapeptide repeats (PAAK, PKAK, PAKK and KAAK) are present in histone H1 and are known to bind DNA (17). Thus these properties of the HLP_{Mt} and its homology to HU proteins suggests that the HLP_{Mt} could be involved in the packaging of mycobacterial DNA. Our results from South-Western analysis using different DNA templates confirm the DNA binding property of HLP_{Mt}

It is intriguing that HLP_{Mt} despite being an intracellular protein interacts with the immune system. We have localized this protein using immuno-gold electron microscopy technique. The protein is confined in its distribution to the cytosolic compartment of the mycobacterial cell (data not shown). This protein like other intracellular proteins has the potential of being used as a marker of lethal permeability changes leading to the death of mycobacteria *in-vitro*. The release of the protein from mycobacteria would be indicative of the alteration in membrane permeability mediated by candidate chemotherapeutic / immunotherapeutic agents.

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DNA VACCINES
APPLICATION TO TUBERCULOSIS

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Introduction

Infectious diseases kill more than 17 million people each year. Acute lower respiratory infections (4.4 million), tuberculosis (3 million) and diarrhoea (3 million deaths, more than 4 billion episodes / year) are the major killers. Malaria (2 million), hepatitis B (1 million), measles (1 million) and AIDS (1 million) are also important and mortal diseases.

Although effective chemotherapy exists for the treatment of a number of these infections, antibiotics are often expensive and in recent years the wide-scale appearance of drug-resistant pathogens has significantly reduced the efficacy of numerous drugs. It is clear that the only cost-effective way to prevent infectious diseases is by vaccination. For example, the widespread use of the small pox vaccine has led to the global eradication of this disease and WHO estimates that vaccination against diphtheria, tetanus, whooping cough, measles and polio prevents approximately 3 millions deaths a year. Unfortunately, effective vaccines are lacking for a number of very important infectious diseases such as AIDS, malaria and tuberculosis.

Recently a new type of vaccine has been discovered, the so called DNA or polynucleotide vaccine, in which DNA encoding a microbial antigen is used instead of the microbe itself. The experimental results obtained in preclinical models have been very promising so far and it is hoped that genetic immunization will open a new era in vaccinology, with immunologically effective vaccines that could be economically accessible also to developing countries.

Vaccines

Effective vaccines aim to stimulate the humoral and/or the cellular immune response. Antibodies are essential in the protection against extracellular bacteria, and the extracellular stages of viruses and parasites, whereas cell mediated immunity (CMI) reflected by cytotoxic lymphocytes and Th1 type cytokine production is needed to defend the host against intracellular bacteria and the intracellular stages of viruses and parasites.

Vaccines can be divided into three categories (Table 1): vaccines based on whole pathogens, either killed or live attenuated, sub-unit vaccines composed of purified proteins or polysaccharide antigens and finally the DNA or polynucleotide vaccines. Whereas

effective humoral immunity can readily be achieved nowadays with subunit vaccines administered in the appropriate adjuvant (ex. tetanus-antitoxin or Hepatitis B surface antigen in alum), effective generation of strong cytolytic and Th1 cytokine production can only be obtained sofar with live attenuated vaccines, which replicate to some extent in the host. Unfortunately, these attenuated vaccines cannot be administered safely in immunocompromised persons, such as cancer patients and HIV - infected people. Vaccines based on killed whole pathogens do not replicate at all but their efficacy is seriously reduced as compared to the attenuated live vaccines. Adjuvants triggering CMI have proven difficult to develop, and the best experimental ones, such as Freund's Incomplete Adjuvant (FIA), are toxic. So far none has been approved officially for human use. The major advantages of the new DNA vaccines are precisely that they can be administered to immunocompromised hosts and induce strong humoral and cellular immune responses without the need for an additional adjuvant.

TABLE 1

CLASSIFICATION OF VACCINES

1. WHOLE PATHOGENS

-Killed:
Good AB + and CD4+ (Th2) response
Poor CD8+ CTL response, poor memory
Polio (Salk), Rabies, Cholera

-Live-attenuated:
Good AB, CD4+ (Th1) response
Good CD8+ CTL response, good memory
Polio (Sabin), Measles, Mumps, Rubella, Varicella, Typhus
Tuberculosis: BCG,

2. SUBUNIT-VACCINES

Good AB and CD4+ (Th2) responses, CD8+?
New Th1 adjuvants needed

- Proteins
Tetanus, Diphtheria, Bordetella, Pertussis
Hepatitis B, Influenza

- Polysaccharides
Haemophilus influenzae b
Meningococcus, Pneumococcus

3. DNA Vaccines
Strong AB, CD4+ and CD8+ responses and memory

DNA Vaccines

1. Introduction

The general principle of DNA vaccination is surprisingly simple, yet major breakthough of this technology only happened during the last decade. A circular plasmid DNA encoding a microbial protein is injected into an immunocompetent host. The plasmid directly translates in a living cell, the gene is transcribed to RNA in the nucleus and translated to protein by the ribosomes. Finally, the host becomes immunized against a heterologous protein, produced by his own cells. Instead of administering a protein, the genetic information for the protein is injected and the host becomes a factory for the production of the introduced gene products. And the work in the factory is well done, as strong humoral and cellular responses are induced, which are long-lasting and protective.

In 1990 Wolff et al. reported that injection into muscle cells of plamid DNA encoding the bacterial enzyme β-galactosidase could lead to direct gene transfer to the muscle cell (25). Enzymatic analysis revealed the presence of the β-galactosidase in the muscle fibers seven days after the plamid injection. Wolff injected the plasmid DNA in a plain salt solution, and discovered that DNA had not to be protected by a lipid coat as previously thought: the technique of "naked" DNA vaccination was born. In 1992 Tang et al. described the ability of plasmids coated onto gold particles and delivered into the skin by a helium-pressure loaded gene-gun, to drive the expression of foreign protein and stimulate an antibody response [19]. The next year, Ulmer, Donnelly et al. reported that they could protect mice against a lethal infection with influenza A by previous intramuscular immunization with DNA encoding a nucleoprotein of the influenza virus [23]. Remarkably, mice could be protected against the Hong Kong strain of 1968 (H3N2), although they had been vaccinated with the nucleoprotein from a strain isolated in the early thirties (H1N1). Thus vaccination with DNA encoding the highly conserved nucleoprotein of the virus led to heterologous, cross-strain protection against influenza strains with different hemagglutinin and neuraminidase serotypes. Since 1993, numerous papers have reported on the efficacy of DNA vaccines in experimental models of infection with various viral, parasite and bacterial pathogens. But before going into more detail about these applications, some general aspects of the method will be discussed.

2. Mechanism

Genetic or polynucleotide vaccination uses a circular, double stranded bacterial plasmid DNA, in which a strong viral promoter/enhancer region controls the expression of the gene of interest [9]. Widely used is the promoter of the immediate early antigen 1 from cytomegalovirus CMV, but SV40 early promoter and the promoter of the Long Terminal Repeats of Rous Sarcoma Virus RSV have also been used. The gene of interest is followed by the eucaryotic transcription/termination poly-adenylation site of bovine growth hormone, needed for the efficient protein synthesis by the ribosomes.. Finally the plasmid DNA has an origin of replication for *E.coli* (OriE) and a resistance marker

(kanamycin or ampicillin) needed for its replication and selective amplification in *E.coli*, respectively. This bacterial plasmid can not replicate in the eucaryotic host and has no homology with eucaryotic sequences, reducing the chance of homologous recombination and integration to almost zero. Examination of mouse muscle injected with plasmid DNA encoding the luciferase gene has indicated that the plasmid persisted and expressed the luciferase for at least 19 months after injection. The methylation pattern of the plasmid remained in its bacterial form (with methylated adenosines) during this entire period, proving that no replication occurred in the cell (which would have led to unmethylated adenosines). Moreover, the plasmid did not integrate but remained extrachromosomal for the entire period (26).

3. Stimulation of the immune system

DNA vaccination is a potent and easy system to generate strong and long-lasting humoral and cellular immune responses. DNA vaccination does not require adjuvants, because bacterial DNA itself has inherent adjuvant properties, by triggering the production of costimulatory cytokines such as IL-12 (stimulation of NK and CD8+ T cells, switch to Th1), IL-6 (stimulation of B cells) and IFN-γ and IFN-γ (which also bias the response towards the TH1 subset). The specific motif in bacterial DNA that triggers these cytokine responses is a 6-base DNA consisting of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (13).

DNA vaccines stimulate both the exogenous and endogenous antigen presentation pathway. MHC class II restricted T helper responses are induced when professional antigen presenting cells endocytose exogenously derived protein synthesized in muscle or keratinocyte. MHC class I restricted CD8+ mediated cytolytic responses on the other hand are triggered by endogenously derived protein synthesized within the cytosol of the APC itself or leaked into the cytosol due to phagocytic overloading (cross-priming).

As clearly demonstrated by the MHC-restriction in parental to F1 bone marrow chimera experiments, professional antigen presenting cells (dendritic cells) are responsible for the priming of the immune response following DNA vaccination (7). It is not clear for the moment whether direct transfection of the APC with the plasmid or so-called cross-priming phenomena play a role. In a second phase, production of antigen by myocytes (i.m) or keratinocytes (gene gun) amplifies the initiated response. Antigen continues to be produced by these latter cells for weeks to months, which may explain the strong and long-lived immune responses generated with DNA vaccines.

Whereas intramuscular injection of saline solutions of plasmid DNA preferentially triggers a Th1 type response, characterized by elevated levels of IL-2 and IFN-γ and a selective production of antibodies of IgG2 isotype, bombardment of the epidermis with DNA-coated gold particles using a gene gun, preferentially triggers a Th2 type response with significant IL-4 production and antibodies mainly of IgG1 isotype (22). Intravenous and intradermal injections a well as mucosal vaccination (oral, nasal, rectal and vaginal) with DNA entrapped in lipid complexes have also been reported.

4. Safety considerations

So far these DNA vaccines have shown very little side effects. However, some theoretical safety considerations concerning integration, tolerance induction and auto-immunity must be discussed.

As already mentioned before, the risk for integration of the bacterial plasmid into the genome which could be mutagenic or potentially carcinogenic, is very small because plasmid DNA is not replicating, injected myocytes are post-mitotic and keratinocytes are shredded from the skin within a week or so. Random integration may take place, but the chances are very low, as demonstrated in an analysis using a very sensitive PCR technique able to detect 1 copy of plasmid/150.000 nuclei (9).

DNA vaccination of neonates could theoretically induce tolerance, but at least for newborn chimpanzees this is not the case, as they can be readily immunized with DNA encoding HBsAg. Also DNA immunization has been reported to abolish a state of tolerance in HBsAg transgenic mice.

Concerning auto-immunity, no side effects have been reported so far. DNA vaccination has even been described to prevent autoimmunity in two experimental models. With respect to anti-DNA antibodies, the immunizing potential of these purified dsDNA plasmids is very low, as compared to denatured DNA used in experimental models, which has to be coupled with methylated BSA and administered in FCA to be immunogenic (17).

5. Preclinical application to tuberculosis

DNA vaccination has been used in a number of preclinical models (Table 2) and very promising results have been obtained, for HIV and malaria (5, 10). It is impossible to discuss all the models in detail and we will only address the subject of tuberculosis.

Tuberculosis remains a major health problem worldwide, with an estimated annual incidence of 8 million new cases and an annual mortality of 3 million people. Combination chemotherapy is very effective to cure this disease, but unfortunately the treatment is long expensive and requires stringent compliance to avoid the development of multi-drug resistant forms. The only tuberculosis vaccine currently available is an attenuated strain of *M.bovis*, termed Bacille Calmette-Guérin or BCG. BCG continues to be widely administered to children in developing countries (WHO/EPI estimate of 100 million doses each year), yet its efficacy remains controversial, particularly against the pulmonary forms of the disease in adults (3). Clearly the development of a more effective vaccine could be an effective solution to the global threat of tuberculosis. As alternatives to BCG, there are several experimental approaches to tuberculosis prophylaxis, including rationally attenuated *M.tuberculosis*, recombinant BCG overexpressing *M.tuberculosis* antigens, protein sub-unit vaccines in appropriate TH1 type adjuvants and DNA vaccines.

A major limitation for all these new approaches is the fact that the protective antigens for tuberculosis are still not precisely defined. The prevailing hypothesis is that they reside within the secreted or exported proteins of the bacillus and this has been supported recently by observations in mice and guinea pigs. Thus immunization with whole *M.tuberculosis* culture filtrate (rich in these exported proteins) can induce significant protection against subsequent challenge with the tubercle bacillus (1, 11). However, these culture filtrates are composed of more than 400 different proteins (P. Andersen, personal communication) and it is not clear whether all are equally important. It is precisely in this context that DNA vaccination may play a crucial role in the development of a better tuberculosis vaccine, as this technique offers a simple method to define the actual protective antigens of *M.tuberculosis*. As discussed earlier, DNA vaccination does not require protein purification and furthermore intramuscular vaccination leads to the generation of strong cellular immune responses with Th1 cytokine profile. It is precisely this type of immunity in which infected macrophages are activated in their bactericidal activity by Th1 cytokines such as interferon gamma or lysed by specific cytotoxic T cells, that should be induced by a good TB vaccine. So far, three types of antigens have been tested as DNA vaccines.

Antigen 85

A major protein component of mycobacterial culture filtrate is the so-called Ag85 complex, a 30-32 kDa family of three protein, Ag85A, Ag85B and Ag85C (24). The bacteriostatic drug isoniazid (INH) enhances the expression of this Ag85 complex in *M.tuberculosis* culture filtrate and recently it has been described to possess enzymatic trehalose-mycolyl-transferase activity (2). Ag85 complex induces strong T cell proliferation and IFN-γ production in most healthy individuals infected with *M.tuberculosis/M.leprae* and in BGC vaccinated mice and humans (14). On the other hand, tuberculosis and lepromatous leprosy patients show decreased cellular immune responses, but increased antibody production to Ag85. Hence, Ag85 could be considered a promising candidate as a protective antigen and using the technique of DNA vaccination, we have been able to prove this hypothesis. Mice vaccinated with plasmid DNA encoding the 85A component of the Ag85 complex, were found to generate robust specific Th1-type helper T cell responses and CD8-mediated cytotoxic T cell responses and could be protected in a mouse model to aerosol or intravenous *M.tuberculosis* challenge (12, unpublished). Vaccination with plasmid DNA encoding the Ag85B but not the Ag85C component was also found effective for generating strong Th1 and CD8+ mediated immune responses (16). Recently, promising results were also obtained in aerosol challenged guinea pigs, immunized with Ag85A DNA (I. Orme *et al.* personal communication).

A major problem with the BCG vaccine is that it induces a positive skin test to PPD and thus interferes with this test in diagnosis of infection. Interestingly, DNA vaccination does not seem to induce delayed type hypersensitivity reactions, despite induction of specific antibodies and lymphoproliferation (unpublished results).

Another advantage of a DNA vaccine encoding Ag85, is that it is very effective in inducing strong CD8+ mediated cytotoxicity, whereas infection with *M.tuberculosis* or vaccination with BCG induce very little AG85 specific CTL activity (8). This is very important in the light of the crucial role that CD8+ T cells play in protection against TB.

The phosphate-binding PstS antigens

DNA vaccination with another culture filtrate antigen, the 38 kD PstS-1 homologue involved in phosphate binding, was described by Vordermeier and his colleagues to result in some degree of protection in mice challenged intraperitoneally or intravenously with *M.tuberculosis* (27). Recently, our research group at the Pasteur Institute in Brussels has performed a comparative analysis of the three PstS homologs (PstS-1, PstS-2 and PstS-3) present in the *M.tuberculosis* genome, and protection against i.v. challenge, comparable to Ag85A DNA or BCG-vaccination could be found in spleen and lungs from C57BL/6 mice vaccinated with the PstS-3, but not with the PstS-1 and only modestly with the PstS-2 DNA (manuscript in preparation).

Cytoplasmic hsp stress antigens

Finally, Silva, Lowrie and their colleagues have reported on DNA vaccination using genes encoding heat shock proteins. Vaccination of mice with DNA encoding the 65 kD hsp from *M.leprae* could induce significant protection in spleen and liver from outbred Parkes mice against intraperitoneal challenge with *M.tuberculosis*. (21). However, the ip.model has its limitations, because only 1% of the bacteria are found in the lungs after this type of challenge, which puts some doubt on the clinical relevance of these results. DNA vaccination with mycobacterial hsp65 probably induces cross-reactive immune responses against self hsp60, as indirectly demonstrated in a model of adjuvant induced arthritis (18). Plasmids expressing hsp 70, the proline rich 36 kD antigen and ESAT-6 also show some, albeit very modest, efficacy in this model (15). Recently, Bonato *et al* have shown in transfer studies that CD8+ CD44+ T cells confer most of the protection (4).

In conclusion, DNA vaccination for TB holds a strong promise for the future. First of all the technique can be used for the definition of the real protective antigens of tuberculosis. Next, the vaccination is easy, does not require protein purification or adjuvants and it is cheap. DNA vaccines do not require a cold chain for their conservation, which is an additional bonus for a vaccine destined in the first place for developing countries. DTH reactions may be avoided and finally, powerful CMI responses of Th1 type are generated whih protect mice and guinea pigs following intravenous and aerosol challenge.

6. Future of DNA Vaccines

So far, DNA vaccination has proven to be a universally applicable technique, that can be efficiently used in the following vertebrate species: fish, bird, mouse, rat, guinea pig, rabbit, ferret, cow, sheep and the non-human primate rhesus monkey and chimpanzee. With respect to application in humans, a number of phase 1 and phase 2 trials have started recently for HIV, malaria, hepatitis B, herpes and influenza, but no results have been published so far (6, 20). At the rate DNA vaccines are currently moving into clinical trials, answers about their true worth should however begin to surface during the next few years. As for a DNA based tuberculosis vaccine, as promising as the experimental results may be, in practice things are not going to be easy. A serious commitment of official health organizations such as WHO and of governments worldwide will be essential, because it is particularly in developing low-income countries that a better tuberculosis vaccine is needed. The relative ease with which these DNA vaccines can be manufactured without sophisticated protein purification technology, as well as the stability of these vaccines which do not require a cold chain for their conservation, are major advantages of this type of vaccine and hold promise for a new era of vaccinology in which developing countries in the South would become self sufficient in their own vaccine production.

TABLE 2
PRECLINICAL MODELS TESTED SO FAR
(Incomplete)

1. Viral infections

Influenza: Nucleoprotein, Hemagglutinin
Lymphocytic chorio meningitis virus LCMV
Human Immunodeficiency Virus: env, rev, gag/pol
Herpes Simplex Virus: HSV1
Bovine Herpes Virus
Hepatitis B: surface Ag
Hepatitis C
Rabies
Papilloma virus
Ebola virus

2. Bacterial infections

Tetanus: toxin C fragment
Bacillus thuringiensis
Mycobacterium tuberculosis: Ag85, hsp65, PstS1
Mycobacterium bovis: MPB 83
Salmonella typhi: OmpC porin
Mycoplasma pulmonis

3. **Protozoal infections**
   Malaria: CSP/HEP17
   Leishmaniasis: gp63

4. **Helminth Infections**
   Schistosoma japonicum: paramyosin

5. **Tumors**
   Carcino-embryonic-antigen: colon, breast, NSLCM
   B cell lymphoma idiotype

6. **Auto-Immunity**
   Experimental Rheumatoid arthritis: hsp65
   Experimental auto-immune encephalitis: vß8.2 TCR

7. **Allergy**
   Shift from Th1 by i.m. vaccination:
   house dust mite

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MYCOBACTERIAL PROTEIN (30/71 KDA) AS SUBUNIT VACCINE AGAINST EXPERIMENTAL TUBERCULOSIS.

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Introduction

Resistance to mycobacterial infections is mediated by a subpopulation of specifically activated T-lymphocytes against selected 'protective antigens' of mycobacteria (1). Recent studies have shown that the protein antigens of mycobacteria, specifically those associated with cell wall peptidoglycan or secreted into growth media by actively growing cells contain the immunoprotective epitopes (2-4). These proteins are known to be recognised by T-lymphocytes at an early stage of infection, thereby playing a protective role against the disease (5). Recently, several reports have shown the role of purified protein antigens in inducing protection against tuberculosis (5,6). However, further work is required to identify the immunoprotective antigens and their protective potential in an appropriate adjuvant system. Hence, studies were carried out to evaluate the immunoprotective role of 30kDa secretory and 71 kDa cell wall proteins, the major immunoreactive proteins of *M. tuberculosis* in different adjuvant systems.

Materials and Methods

Animals and mycobacterial cultures

NMRI (Inbred) mice originally obtained from CRI, Kasauli, India were maintained in the animal house of the department *M. tuberculosis* $H_{37}Ra$, *M. tuberculosis* $H_{37}Rv$ and *M. bovis* BCG were obtained from NCTC, London and maintained on Lowenstein-Jensens medium and modified Youmans medium.

Isolation of antigens

71 kDa cell wall and 30 kDa secretory proteins were isolated and purified as described previously (7,8)

Preparation of liposomes and PLG-MPs

Frozen-thawed multilamellar vesicles (FT-MLVs) were prepared by hydration of lipid film (8), whereas PLG-MPs were prepared by the method as described earlier (9).

Immunization of animals

The mice (in groups of 20 each) were immunized subcutaneously on day 0,7 and followed by an intramuscular injection on day 14 with 71 kDa/30kDa proteins using

FIA/liposomes/PLG-MPs as adjuvants. Another group of mice were immunized with $1x10^6$ colony forming units (CFU) of BCG vaccine intravenously (i.v.). Control animals were sham-immunized with saline in adjuvant.

Immune responses

4-5 animals from each group were exsanguinated at weekly intervals and their spleens were removed aseptically to study the cell mediated immune responses. T cell proliferation assay was performed as described previously (7,8). The amount of cytokines (IL-2 & IFN-γ) present in the culture supernatants were quantified by enzyme linked immunosorbant assay using commercially available kits (ELISA) from Genzyme Immunobiologicals.

Protection studies

Control and immunized animals were challenged 8 or 16 weeks p.im. intravenously with LD_{50} i.e. $3x10^7$ CFUs of *M. tuberculosis* $H_{37}Rv$. The protection induced was evaluated by monitoring the survival rates and CFU enumeration in the infected organs at 30 days p.im.

Mechanism of immunoprotection

Mechanism of protection provided by 71 kDa/30 kDa proteins was studied by adoptive transfer of various immune cells (CD3+, CD4+ and CD8+) into irradiated recepient mice.

Statistical analysis

Data of proliferative responses and CFU enumeration were analyzed by student's t -test, whereas, chi-square was used for the analysis of protection data.

Results

Immunogenicity of purified 71 kDa/ 30 kDa proteins

Significantly high level of in vitro lymphocyte stimulation was induced by both 71 kDa and 30 kDa proteins in mice immunized with 71 kDa-FIA and 30 kDa-FIA respectively (Fig. 1a,b) and these responses were better than that obtained in BCG vaccinated mice (data not shown). Further, in the 71 kDa-PLG immunized group, the level of stimulation at fourth week p.im was significantly greater ($p<0.01$) as compared to 71 kDa-FIA immunized animals and remain sustained till the sixteenth week p.im (data not shown). A significant ($p<0.01$) T cell reactivity was observed in 30 kDa-lip immunized animals which was maximum at 3rd week p.im and comparable to that produced in 30 kDa-FIA immunized animals (Fig.1b). Significantly high levels of IL-2 and IFN-γ were found in culture supernatants of T - lymphocytes obtained from 71 kDa/30 kDa immunized mice as compared to BCG vaccinated group and no cytokines were detected in unstimulated cultures. In 30 kDa-lip immunized animals, levels of these cytokines

were comparable to that produced in mice immunized with 30 kDa-FIA. However, 30-40% higher levels were observed in the 71 kDa-PLG immunized group as compared to 71 kDa-FIA immunized animals which remain sustained till eighth week p.im.

Table 1

M. tuberculosis $H_{37}Rv$ enumerated from the target organs of 71 kDa-PLG-MPs, 71 kDa-FIA, BCG and control mice challenged at eighth week p.im.

Group	Viable Counts		
	Lung	Liver	Spleen
71 kDa-PLG-MPs	$1.88\pm0.18\times10^{6***!!!++}$	$1.52\pm0.42\times10^{5**!!!NS}$	$1.35\pm0.19\times10^{5***NS}$
NS-PLG-MPs	$4.25\pm0.52\times10^{8}$	$3.36\pm0.2\times10^{7}$	$2.78\pm0.34\times10^{5}$
71 kDa-FIA	$2.59\pm0.146\times10^{6***!!!}$	$1.21\pm0.03\times10^{5***!!!}$	$1.10\pm0.45\times10^{5*NS}$
NS-FIA	$3.48\pm0.92\times10^{8}$	$3.01\pm0.67\times10^{6}$	$2.86\pm0.52\times10^{5}$
BCG	$3.97\pm0.43\times10^{7}$	$3.22\pm0.76\times10^{6}$	$1.84\pm0.68\times10^{5}$

Results are presented as mean of 4-5 animals of duplicate experiments.

*** $p < 0.001$; ** $p < 0.01$, wrt corresponding control.
!!! $p < 0.001$, wrt to BCG immunized group.
++ $p < 0.01$, wrt to 71 kDa FIA immunized group.
LD_{50} - Median lethal dose (3×10^7 CFUs/mouse).

Protective efficacy of 71 kDa/ 30 kDa proteins

Eighth week p.im. animals were challenged with LD_{50} dose of *M. tuberculosis* $H_{37}Rv$ and protection was monitored on the basis of survival rates and CFU enumeration. 71 kDa induced equivalent protection (Fig. 2) on the basis of survival (90%) with both the adjuvants i.e. FIA and PLG-MPs which was significantly higher in comparison to BCG (70%, $p<0.5$) and controls (40-50%, $p<0.001$). A survival of 86% was seen in 30 kDa-FIA group which was comparable to that seen in 30 kDa-lip immunized animals. The number of bacilli recovered from the infected organs of 71 kDa-FIA and 71 kDa-PLG immunized groups exhibited a significant decrease in bacterial load as compared to controls as well as BCG vaccinated animals (Table 1). Similarily, a significant decrease in viable bacilli was seen in the lungs, liver and spleen of 30 kDa-FIA and 30 kDa-lip immunized animals as compared to control (Table 2). In animals immunized with 71 kDa-PLG and challenged at sixteenth week p.im; the protective efficacy was sustained (85% survival) but exhibited a marked decrease (70% survival) in 71 kDa-FIA immunized animals.

Table 2

Viable *Mycobacterium tuberculosis* $H_{37}Rv$ obtained from animals immunized with (30 kDa-FIA, 30 kDa-liposome and BCG vaccinated) and challenged with LD_{50} of *M. tuberculosis* $H_{37}Rv$.

Immunized Group	Colony forming units (mean ± SD)		
	Lung	Spleen	Liver
30 kDa-FIA	$2.8\pm0.4x10^{5++}$	$1.81\pm0.8x10^{5++}$	$7.23\pm1.1x10^{5++}$
30 kDa-liposome	$3.44\pm1.01x10^{6+}$	$1.99\pm1.5x10^{6*}$	$3.2\pm1.6x10^{6+}$
BCG vaccinated	$3.3\pm0.36x10^{6+}$	$2.6\pm0.28x10^{6+}$	$3.3\pm0.3x10^{7**}$
Liposome (control)	$7.21\pm0.001x10^{7}$	$6.20\pm1.2x10^{7}$	$8.31\pm0.98x10^{7}$
NS-IFA	$9.27\pm0.021x10^{7}$	$5.83\pm0.11x10^{7}$	$7.48\pm1.4x10^{7}$

*Significant at $p<0.025$ level; ** not significant; + significant at $p<0.01$ level; ++significant at $P<0.001$ level. IFA, in complete Freund's adjuvant ; BCG bacille Calmette-Guerin; NS-IFA, normal saline in IFA.

Mechanism of protection

The recepient mice, passively transferred with CD4+ and CD8+ from 71 kDa/30 kDa immunized mice and challenged with LD_{50} of *M. tuberculosis* $H_{37}Rv$ exhibited greater survival (66-73%) which was further enhanced in case of total spleenocytes (86%) consisting of dendritic cells, natural killer cells, macrophages in addition to CD4+ and CD8+ T-lymphocytes.

Discussion

Recent studies carried out in our lab have shown 71 kDa cell wall protein and 30 kDa secretory protein of *Mycobacterium tuberculosis* as the most immunoreactive proteins exhibiting significant T cell reactivity. The present study was undertaken to investigate the immunoprotective activity of 71 kDa and 30 kDa proteins against experimental tuberculosis.

Animals immunized with pure 71 kDa/30 kDa proteins in FIA exhibited enhanced cellular responses which were higher than that of BCG vaccinated animals. Further, cytokine profile of 71 kDa / 30 kDa activated T-lymphocytes suggested efficient activation of Th1 subset of CD4+ T lymphocytes which are important mediators of protective immunity (6). These findings are in agreement with other workers who have shown that

protein antigens of mycobacteria activate the TH1- type T cells which secrete IL-2 and IFN-γ (6,10). The immune responses are known to be influenced by the adjuvant used and they possess some selectivity for the induction of a particular immune response. In several studies, liposomes have been shown to induce strong cellular immune responses and provide a longer duration of antigen stimulation (11,12). Synthetic biodegradable polymers have also gained considerable attention as adjuvants because of their potential for long term controlled release of proteins (13,14). Immunization with 30 kDa-lip resulted in significant T cell responses skewed towards Th1 and comparable to that seen in 30 kDa-FIA immunized group thus suggesting that liposomes can effectively replace FIA. Our findings are in accordance with earlier studies, in which, liposomes have been used as adjuvants for mycobacterial antigens (15,16). However, PLG-MPs used as carrier in the present study for 71 kDa protein induced higher and prolonged TH1 responses in comparison to FIA (Fig.1a). These results are in accordance with previous report on immunogenicity of 38 kDa mycobacterial protein using PLG-MPs as adjuvant in comparison to FIA (17).

Animals immunized with 30 kDa-lip and 30 kDa-FIA exhibited significant and comparable protection against a sublethal challenge of *M. tuberculosis* H_{37}Rv 8 weeks p.im as demonstrated by increased survival and decreased viable counts. This finding suggests that FT-MLVs can be successfully used for various mycobacterial antigens. However, as the protective immunity provided by 30kDa-FIA declined with time and immunoreactivity of 71 kDa protein decreased on encapsulation in liposomes (data not shown), another PLG-microparticles (PLG-MPs) based antigen delivery system was used. 71 kDa in PLG-MPs provided a long term protection (Fig.2) which was sustained till the sixteenth week p.im. (data not shown) and was significantly higher than that seen in 71 kDa FIA or BCG immunized groups. Our results suggest that PLG-MPs efficiently induce long term protective responses by slow release of antigen. Further microparticles have also been approved for oral or intranasal administration of drugs/antigens (18). The protective effect of both 71 kDa and 30 kDa proteins was found to be mediated by the co-operative effect of CD4+ and CD8+ T cells suggesting mycobacterial antigen presentation in association with of MHC class I and II molecules.

In brief, mycobacterial 71 kDa cell wall associated and 30 kDa secretory proteins having protective efficacy comparable / better than BCG vaccine are suitable candidates for subunit vaccine development against tuberculosis.

Future plan of work

To further evaluate the potential of these proteins for the development of subunit vaccine, immunoreactivity of these proteins would be examined in animals of different strains exposed to environmental mycobacteria. Further, these aspects will be examined in population of different genetic constitution (HLA types), exposed to different environmental mycobacteria and having different PPD / tuberculin reactivities.

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STUDIES ON THE IMMUNOPATHOLOGY OF TUBERCULOSIS

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In this presentation, two aspects concerning the immunopathology of tuberculosis at the microanatomical level will be considered. The material for this presentation is drawn from our studies on tuberculous lymphadenitis (450 cases) and skin tuberculosis (270 cases).

The first question to be considered is 'when is it necessary to demonstrate the components of *M.tuberculosis* in the tissues?' The second is the question of the involvement of humoral immune factors associated with some of the histological manifestations of tuberculosis.

Demonstration of the components Of *M.tuberculosis*

There are three clinical situations where it is necessary to visualise the components (either as antigens or as nucleic acids) of *M.tuberculosis* in the affected tissues.

1. Establishment of Diagnosis

Histopathologically, the signature of tuberculosis is usually unmistakable. The usual picture of a well-formed, epithelioid cell, giant cell, lymphocyte, plasma cell granuloma with a central caseation necrosis is often enough to confirm the clinical diagnosis of tuberculosis in an endemic country.

However, in about 25% of the cases, variant forms may be seen. These may include a well-formed, non necrotic, epithelioid cell granuloma at one end and a poorly-organised granuloma with vacuolated macrophage and necrosis without caseation at the other end. In such cases, demonstration of bacilli either by Ziehl-Neelson (Z-N) staining or culture for *M tuberculosis* would be helpful in establishing the diagnosis. Nevertheless, in about 40% of these cases, bacilli are usually not seen using Z-N staining and 25% of these are culture negative for *M.tuberculosis*. It is likely that demonstration of the bacillary components in such cases using probes which are specific for the organism will help the pathologist to arrive at the diagnosis of tuberculosis in such atypical presentations.

2. Establishment of the aetiology of granulomatous inflammation:

Identification of the nucleic acids of *M.tuberculosis* in granulomatous lesions has helped to identify the aetiological agent in two conditions.

a. Tuberculids: Erythema induratum and papulonecrotic tuberculids occurring in the skin are rare conditions traditionally attributed to a tuberculous aetiology. A caseating granuloma is often seen histologically and this has been considered to be a manifestation of tuberculous hypersensitivity with the primary lodgement of the bacilli elsewhere in the body as *M.tuberculosis* cannot be cultured from these lesions.

Recently, the tuberculous aetiology of these lesions has been confirmed by Degitz and his colleagues (1993). Using a 383bp fragment of the mycobacterial gro EL gene amplified by primers TB1 and TB2, the tuberculous actiology of this granulomatous lesion has been identified by these workers.

b.Sarcoidosis: This forms an important differential diagnosis both clinically and histologically while a diagnosis of tuberculosis is actively considered. The actiology of this condition has long remained a matter of speculation. However, several investigators have demonstrated *M.tuberculosis* nucleic acids in these lesions (reviewed in Mangiapan and Hance, 1995). It is possible that the granulomatous inflammation in sarcoidosis is an allergic manifestation caused by fragmented *M.tuberculosis*.

3. Establishment of resolution

Demonstration of the presence of *M.tuberculosis* antigen(s) may be of use in one more clinical situation. It is sometimes seen that in patients with tuberculous lymphadenitis, the lymph node does not decrease in size completely after a full course of anti tuberculous therapy (ATT). In such situations, biopsy of the node has shown that while a residual granuloma is still present, *M.tuberculosis* is neither seen on Z.N staining nor can it be cultured. Demonstration of *M.tuberculosis* antigen will be of use in deciding whether the granuloma is still active and therefore require a further course of ATT.

Experiments were therefore conducted in the guinea pig to observe the resolution of granuloma *vis a vis* intact bacilli and *M.tuberculosis* antigen. Killed *M.tuberculosis* (2×10^7) was injected intradermally and groups of four animals were sacrificed at various time points from 6h to 12 weeks and the injected site was removed. In another experiment, live *M.tuberculosis* (2×10^7) was injected intramuscularly and the animals were sacrificed from 4 to 44 weeks and their spleen, liver and lung were obtained. These, along with the skin specimens were evaluated for the presence of granuloma, acid fast bacilli (AFB) and *M.tuberculosis* antigen(s). Tuberculous antigen was demonstrated using polyclonal anti *M.tuberculosis* antiserum in an indirect immunoperoxidase method.

In all the four organs sites examined, stainable bacilli were the first to disappear followed by *M.tuberculosis* antigen and finally only the granuloma resolved. Further, it was found that the splenic culture of the infected guinea pig for *M.tuberculosis* became

negative long before the disappearence of the antigen. It is therefore concluded that the presence of granuloma in the absence of antigen in a clinical specimen from a residual lesion probably indicates that the granuloma is in the resolving stage and that the patient may not require further ATT.

Whither B cell responses in tuberculous granuloma?

A detailed analysis of the cellular profile of the histology of tuberculous lymphadenitis (TBL) and cutaneous tuberculosis (CTB) revealed the following:

1. A spectrum of responses varying from a well-organised, non necrotic epithelioid cell granuloma (Hyperplastic) through a caseating, epithelioid cell granuloma (Reactive) to a poorly-organised, macrophage granuloma with noncaseating necrosis (Hyporeactive and Nonreactive) was observed.

2. In cutaneous tuberculosis, the histology correlates with the clinical spectrum of verrucosa cutis, lupus vulgaris and scrofuloderma. Verrucosa cutis is characterised by a non necrotic, epithelioid cell granuloma while in scrofuloderma, necrosis is a hall mark of its histology. In lupus vulgaris, minimal necrosis was seen in 9 out of 127 cases.

3. Plasma cells and B cells were found in moderate numbers in all these granulomata; these were seen more often when there was necrosis (Table 1).

4. There were no differences in the response to purified protein derivative (RT23) at 72 hours amongst the various histological groups within TBL or within CTB (Table 2).

TABLE 1: DISTRIBUTION OF PLASMA CELLS AND B LYMPHOCYTES IN TUBERCULOUS LYMPHADENITIS AND SKIN TUBERCULOSIS
(Mean±95% confidence interval)

	TYPE	PLASMA CELLS	B LYMPHOCYTES
LYMPH NODE			
	HYPERPLASTIC	0.7±0.3	6.8±1.2
	REACTIVE	7.5±0.6	12.1±0.9
	HYPO REACTIVE	12.4±2.1	17.3±1.1
	NON REACTIVE	18.5±5.8	19.4±1.5
SKIN			
	LUPUS VULGARIS	4.4±0.9	10.1±1.3
	VERRUCOSA CUTIS	4.0±1.2	13.1±8.1
	SCROFULODERMA	16.2±1.6	18.4±5.6

TABLE 2: MANTOUX REACTION AT 48h TO PPD

TYPE	<10mm	>10mm	MEAN	95% C.I.
LYMPH NODE				
HYPERPLASTIC	9	55	25.5	20.8-28.3
REACTIVE	24	135	26.8	24.1-29.5
HYPOREACTIVE	8	41	25.4	22.8-27.9
NONREACTIVE	4	7	27.3	15.2-34.3
SKIN				
LUPUS VULGARIS	11	72	16.9	14.9-18.9
VERRUCOSA CUTIS	11	61	17.9	15.7-20.1
SCROFULODERMA	2	17	19.2	15.4-23.0

It is possible that these represent a polarisation of the T lymphocyte responses into Type 1 and Type 2 patterns as has been demonstrated in leprosy lesions (Yamamura *et al*, 1991; Salgame *et al*, 1991). The patterns of T cell cytokine response from human peripheral blood mononuclear cells to PPD have been studied (Del Prete *et al*, 1991; Barnes *et al*, 1992). A shift from Th1 to Th2 response occurs when the acute stage of the disease leads onto the chronic stage in the lung tissue of a murine model for tuberculosis (Hernandez-Pondo *et al*, 1996).

The cytokine profile of the lymphocytes in human tuberculous granuloma is yet to be characterised fully. It is tempting to argue that the histological spectrum observed in TBL and CTB may represent polarised T cell responses. However, the Mantoux reactions in these patients do not show differences amongst the various histological groups suggesting that a clear cut polarisation as seen in leprosy lesions may not be demonstrable in tuberculosis though this needs to be looked at in greater detail.

The presence of moderate numbers of B cells and plasma cells in lesions where there is necrosis needs to be explored further. A number of possibilities exist to explain their presence and some of them are discussed here.

The plasma cells arising from B cells can produce antibodies and along with the fragmented *M.tuberculosis* antigen can form *in situ* immune complexes. These then can not only initiate but also determine the type of granuloma formed (Spector and Heesom, 1969, Ridley *et al*, 1982). Futher, Campa *et al* (1989), have shown that anti idiotypic B cells against *M.tuberculosis* can modulate both granuloma formation and delayed hypersensitivity reaction to PPD. Antigen-specific B lymphocytes are potent antigen presenting cells. It is known that B cells presenting antigen to naive T lymphocyte can result in tolerance whereas if they present antigen to sensitized T cells, they can be immunized (reviewed in Matzinger, 1994).

Recommendations

In view of the foregoing, three major areas of research concerning the immunopathology of tuberculosis are recommended.

1. Development of reagents to identify *M.tuberculosis* antigen(s)/nucleic acid(s) in tissues suspected to be of tuberculous pathology though with a caveat. It is advisable to use these reagents **only** when there is a *prima facie* case for doing so. In other words, the lesion should have a granuloma and bacilli not demonstrable either using Z-N staining or by culture for the organism.
2. Delineation of the *in situ* cytokine responses in different histological forms of tuberculosis.
3. Elucidation of the role of humoral immune responses, especially that of the B lymphocytes in the histological manifestations of the disease.

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HOST RESPONSES TO *MYCOBACTERIUM TUBERCULOSIS*

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The disease tuberculosis (TB) occupies an unique status both in emerging and re-emerging disease list even as of today in most geographical regions of this globe. There seems to be an extended relationship between man and mycobacterium from time immemorial to the present days. This is evidenced by the increasing incidence world wide of TB cases along with mounting HIV infection and upsurge of cases of AIDS. Thus TB remains one of the most common infectious disease in man. In turn this scenario of TB, reconfirms and ascertains, a rather complex intertwined partnership between man and mycobacterium. The understanding of this relationship is a science in itself, which has generated a renewed research worldwide solving chronic questions and creating newer ones. Yet there are lacunae in the understanding of the multi facet reactions of human host to *Mycobacterium tuberculosis* (M tb) inspite of outputs from advances in biology, immunology, chemistry, molecular biology and genetics of mycobacteria.

There are many questions to be addressed for planning the control of TB. A few of these questions are:

1. Why is TB a major challenge to epidemiologists, basic scientists, physicians, public health authorities dealing with mycobacteria ?
2. Can we promote prevention of TB with an ideal vaccine which stimulates the right type of immune cells to bring about control and killing of the bacilli ?
3. How does one arrest reactivation of TB ?
4. Is molecular dissection possible between primary TB pathogenesis and reactivation TB pathogenesis?
5. What are the virulence factors of mycobacteria in the light of molecular level ?

The recent attempts at molecular approach to mycobacterial pathogenesis have partially attempted to answer some of these questions. Since the discovery of tubercle bacilli by Robert Koch in 1882, its interactions with mammalian hosts has been studied in clinical and laboratory settings. The variations and heterogeneity of clinical presentations, course of disease with or without treatment, highly variable mycobacterial and immunological profiles as studied in the diagnostic laboratory have reaffirmed the complexity of TB. The highly complex nature of the mycobacterial cell wall coupled with the equally complex host responses has made TB "a disease of the past returning to haunt the future".

In general *Mycobacterium tuberculosis* has a programmed route and fate in the human body. The bacteria which reach the lung are taken up by alveolar macrophages and the M.tb are able to grow within. Only activated macrophages can kill M.tb. The initial survival within the macrophage is made possible due to special lipid coat of M tb. But for the unusual mycobacterial cell wall, the scenario of TB would have been very different. Delay in processing of the bug, and programmed release of various antigens are responsible for initiating the immune responses both humoral and cell mediated. Macrophages that ingest M tb, process the bacterial antigens in ways that elicit both T helper(CD4+) cell response and cytotoxic T (CD8+) cell response. Th2 subset of CD4+ T cells stimulate antibody production which are practically of no use to control the disease from the host's point. On the contrary the released antibodies can hatch on to the respective antigens to form mycobacterial immune complexes. These may play a major role in immunopathogenesis of disease process and bring on many immune mediated pathology such as arteritis and arachnoiditis in neurotuberculosis.

The Th1 subset of CD4+ cells, release interferon gamma (IFN-gamma) which aid in activation of macrophages to bring about microbial killing. The CD8+ T cells, may kill macrophages that have been unsuccessful in killing bacteria, thus releasing the bacteria to be ingested by activated macrophages.Too large a lesion non-manageable by a macrophage, is walled off in the tubercle, the pathognomonic tissue reaction of TB. The tubercle has a characteristic anatomic and immune cell architecture which consists of central caseation necrosis surrounded by layers of epitheloid cells, CD4+ T cells, giant cells and fibroblasts with attempted fibrosis and an optional calcification. Bacteria can survive in these tubercle for decades, only to reemerge later during an event of immunosuppression. This phenomenon is termed reactivation of TB. In the event of escape of M tb from tubercle due to undue liquefaction of the central caseum which in itself promotes active growth and accentuated metabolism from an inert dormant M tb present in the thick caseum and can lead to dissemination of TB. If the M tb escapes from lung, they can spread to any organ of the body and lead to systemic, disseminated and even fatal form of TB.

The immune host response that occur during tuberculous infection are an interplay of two major mechanisms, namely cell mediated immunity (CMI) and delayed type hypersensitivity (DTH). The term CMI refers to antigen specific host responses that lead to activation of macrophages and T cells to kill the M tb. DTH refers to antigen specific and non specific responses that lead to destruction of host tissue containing mycobacteria. It is this destruction of host tissue in settings such as central nervous system which is the major contributor of morbidity in neurotuberculosis.

These immunological and other host responses triggered by mycobacterial antigens including heat shock proteins, antibodies, immune complexes and cytokines decide on the heterogeneity of tissue reactions to infecting M tb, the corresponding clinical presentation, disease progression, the availability of mycobacterial and/or host factors for use in diagnosis of TB, be it microbiological, immunological or molecular and

the response to therapy both chemo and immuno modulative therapies. The events which follow the interaction between M tb and the host will depend on whether the host is immunologically competent, immunologically compromised or immunologically inert.

The past decade has witnessed an explosion of information and insight into immuno-regulatory proteins namely cytokines, heat shock proteins and autoimmunity, and molecular approaches to virulence of mycobacteria. These have contributed to better understanding of mycobacterium man interactions.

Cytokines

These proteins are aptly defined as small, non structural, intracellular, regulatory proteins that mediate a multiplicity of immunologic as well as non immunologic biological functions. They play an important role in immunoregulatory effects in host responses to M tb infection. A cascade of these factors, chief of them being Interferon gamma and Tumour necrosis factor alpha, along with others such as Interleukins (IL) 1, 6, 8, 10 and 12, transforming growth factor beta (TGF beta) are released from a variety of immune reactive cells like monocytes/macrophages, CD4+ T cells, epitheloid cells and giant cells. These mediate many of the clinical manifestation of the disease TB such as weight loss (cachexia) and fever. Some of the cytokines decide on the predominant inflammatory cell response and immune cell namely Th1/Th2 cell responses and granuloma formation. This new insight into various cytokines and their respective role in pathogenesis and control of tuberculous process, has resulted in better understanding of intricacies of pathogenesis of TB. The availability of these double edged proteins, and their proportions in a given case of TB may now explain in part the clinical, immunological and pathological heterogeneity of a tuberculosis patient be it at initial presentation or during disease progression. This has opened the doors to the possibility of role of immunomodulation in addition to chemotherapy of TB.

Mycobacterial heat shock proteins/stress proteins and its role in immunopathogenesis and autoimmunity

In general all microbes respond to sudden increase in temperature (heat shock) by increasing the rate of synthesis of a small number of highly conserved proteins called HSPs. The response is termed heat shock response. These are also termed stress proteins. A variety of stress such as environmental stresses, nutrient deprivation, exposure to heavy metal ions, chemicals can induce release of stress proteins from host. The host defenses against mycobacteria can in turn induce mycobacterial HSPs.The mycobacterial HSPs are of five types: HSP90, HSP70, HSP60, HSP10 and other low molecular weight HSPs. These HSPs play an important role in host - mycobacterial interaction. The molecular analysis of the genes encoding for these special mycobacterial proteins revealed the surprising result that many of the proteins have striking similarity to amino acid identities of other microbial and host stress proteins. From the mycobacterial point of view HSPs are important for normal cellular growth and functioning and also required to face the onslaught of hosts professional phagocytosis and other effects of the recurrent fever seen in TB. From the hosts point of view, expression of pathogen's or host's HSP on the surface of the macrophages may be an early indication of infection and may serve as a general immuno surveillance system for infection. The

immune response to HSP can be a protective immune response to the host. But at other times can be detrimental to the host by initiating an autoimmune process. The antibodies produced against the mycobacterial HSP on cell surface initiate a series of auto immune process. T cell elicited by mycobacterial HSPs could recognise and lyse autologous stressed' macrophages. These antibodies and T cells, directed to stress proteins can cross react with antigens on surface of human epidermal cells, cytokeratin, lactoferrin, transferrin and HLA DR beta molecules. In several animal models, of auto immunity there is direct evidence that immune responses to HSP 60 is involved in autoimmunity. In humans, the relationship between HSP and autoimmunity is controversial. Further studies are needed to study the regulation, expression, function of HSPs and their role in immune responses, and to distinguish beneficial from detrimental effects. This information may open up challenging angles for immunotherapy and prevention of TB.

Preliminary studies in CSF of TBM cases have revealed immune responses to heat shock protein (65 Kda antigens).

Histochemical methods using ML30 monoclonal antibodies have localised these antigens in tissues of central nervous system (astrocyte) damaged by M tb.

Mycobacterial virulence factors

A. Molecular look

In the past, several animal models have been designed to study pathogenesis of TB and virulence factors, in mice, guinea pigs, rabbits and monkeys. The evolving science of molecular biology and molecular genetics has made possible the dissection of virulence factors at gene level. However, the slow generation time of M tb, its complex cell wall, lack of methods for constructing disruptions in specific chromosomal genes, the poorly working homologous recombination, have impeded advances of understanding M tb virulence factors at molecular level. Yet breakthroughs have occurred in identifying pieces of DNA responsible for cell invasion, survival within phagocytes, drug resistance and proteins for host tissue destruction. This process has now become an ongoing Mycobacterial molecular science. The identification of the gene for specific proteins which trigger the protective immunity in host would go a long way to redesign a better vaccine for the present times.

Conclusion

This complex subject befits a conclusion in a philosophical note. Thus it is apt to re echo the thoughts and statements of discoverer of Tubercle bacilli, Robert Koch. **"The bacillus is not all there is to tuberculosis"**. The above statement is very apt with respect to complexities of host's reaction to M tb infection.

Yet another golden statement of the father of microbiology is: "In the future the battle against plague of mankind (TB) will not just be concerned with an uncertain something but with a tangible parasite about whose characteristic a great deal is known and can be explored". The above statement is so true then and today, as we enter the next millennium.

IMMUNODIAGNOSIS OF TUBERCULOSIS

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It is clear from the recent epidemiological figures that tuberculosis due to *M.tuberculosis* infection is on the rise both in the developing and developed countries. One of the effective methods to control infection is to identify the disease at an early stage for its effective treatment and cure to help the public health measures. The laboratory diagnosis of *M.tuberculosis* plays a pivotal role in early diagnosis of the disease. As the recent methods which are available have limitations in diagnosing the disease early, there is an immediate need to develop a sensitive, specific and a cost-effective assay for the diagnosis of tuberculosis.

The conventional procedure for detection of mycobacteria in clinical samples is based on the demonstration of acid-fast bacilli (AFB) by microscopy and by its routine culture. Although, the method is very reliable it is time consuming. It can take 6 to 8 weeks in culture to account for the growth of AFB. In addition, culture has a low degree of sensitivity and further by using this technique, it is not possible to differentiate between closely related species (Bates, 1979; Wallace et al, 1990). Microscopic detection of mycobacteria is limited by its low level of specificity and it does not allow for the detection of mycobacteria at a concentration below 10^4/ml (Yeager et al, 1967). Mycobacterial species can be identified by methods such as high performance liquid chromatography which can detect 50 different mycobacterial species. However, in such cases also presence of more than 10 bacteria is required in the samples (Butter and Kilburn, 1988). Serological tests using monoclonal antibodies have also been developed, however, these tests have the major limitations regarding their sensitivity with clinical samples.

T Cell Proliferation Assays

(i) Status of purified protein derivative (PPD)

The DTH skin reaction induced by intracutaneus inoculation of PPD is a well recognised, widely used screening test for detection of infection with tubercle bacilli. However, both specificity and sensitivity of PPD testing are compromised (Bass, 1993). The test specificity is reduced due to previous sensitizations by BCG vaccination and environmental mycobacteria (Haslov et al, 1996). Therefore, this test seems to be of little value in endemic countries of Asia and Africa. In the United states, tuberculin skin testing has shown to yield low specificity in pulmonary tuberculosis due to the presence of other respiratory mycobacterial pathogens (Huebner et al, 1992). Further, false-positive results often lead to improper initiation of chemoprophylaxis and overestimate of infection in the community (De March Ayuela, 1990). Lastly, PPD preparations vary widely from batch to batch due to the crudeness in the preparations posing difficulty in standardisation.

(ii) Species specific antigen and peptides

It is well known that species specific immunodominant antigens of *M.tuberculosis* would be the most attractive proposition for use as immunodiagnostic agents. These agents, if developed, would have the ability to select out and identify the disease from a cross reactive sensitization situation in the community.

Daniel et al (1979) employing affinity chromatography isolated "antigen 5" from PPD. This antigen was later found to contain an immunodominant lipoprotein antigen, 38-kDa protein with phosphate-binding activity. "Antigen 5" which contains 38 kDa antigen was found to be no more specific than PPD (Daniel et al, 1982). Recently, Singh et al (1992) cloned the gene of 38-kDa antigen and over expressed the same in *E.coli*. With cell proliferation experiments and r-IFN studies, this over expressed protein recognised 69% of healthy sensitized subjects (Wilkinson et al, 1997).

With the application of molecular biological techniques various synthetic peptides have been found to be effective in eliciting DTH skin reaction in sensitized guinea pigs, mice and human volunteers (Minden et al, 1986; Vordermeier et al, 1992; Ashbridge et al, 1992 Vordermeir et al, 1993). Of all the immunogenic peptides, the C-terminal 350-364 "G" epitope of the 38 kDa antigen was recognised as a specific and a genetically permissive epitope in mice. Further a mixture of eight peptides derived from sequences of 16 - 19 and 38 kDa antigens when used in a T cell proliferation assay, identified individual with sensitivities of 100%, 87% and 82% in untreated patients, treated patients and tuberculin positive controls respectively. (Jursevic et al, 1996). However, the absolute level of r-IFN produced with mixture of peptides were significantly less than that induced by PPD. Synthetic peptides as such are not known to be good cell stimulators because these are not processed by APC (Lanzavecchia and Watts 1994) and in addition, these are known to bind in a relatively small proportion (<10%) of MHC class II molecules of cell surface (O'Sullivan et al, 1990). Therefore, there is a further scope to work with multiantigenic peptide presented in microspheres or liposomes for a greater cell stimulation.

(iii) Antibody based assays

Several antibody based tests for detection of *M.tuberculosis* infection was established by using polyclonal and monoclonal antibodies (Mabs) (Ivanyi et al, 1983; Sada et al, 1983; Kadival et al, 1986; Watt et al, 1988; Cho et al, 1990; Wilkinson et al, 1997) but none of these acquired a widespread support in the diagnosis of tuberculosis. Major problems associated with antibody based tests are their cross reactivity with other mycobacterial strains alongwith their variable sensitivity. Several Mabs have been generated against *M.tuberculosis* (Coats et al, 1981, Daniel et al, 1984; Anderson et al, 1986; Mauch et al, 1988, Cho et al, 1992) but they were either cross reactive with other strains (Cho et al, 1990; Mauch et al, 1988) or less sensitive in the assay (Daniel et al, 1984; Ivanyi et al, 1983; Kadival et al, 1986).

Tuberculosis specific Mabs were extensively applied in competition enzyme linked immunosorbent assay (ELISA) against sera of patients and Ivanyi et al (1985) identified 38, 19-and 16-kDa antigens as major immunogens. They noted that while multibacillary pulmonary tuberculosis showed high levels of anti 38-kDa antibody, antibodies to 16-kDa antigen were noted at high levels in chronic household contacts and in hospital workers (Jackett et al, 1988; Bothamley et al. 1992 a). Chandramukhi et al (1989) noted elevated levels of lipoarabinomannan and 16-kDa antigens in the cerebrospinal fluid of tuberculous menignitis. While a high level of anti 38-kDa antibody and low level of anti 16-kDa antibody was associated with pulmonary tuberculosis with poor prognosis, patients showing high anti-19 kDA titres showed less pulmonary cavitation. Bacteriological relapses were frequently noted in patients with rise in 16-kDa. TB 68 epitope, antibodies. Considering the above observations Bothamley et al (1992 b) opined that simultaneous measurement of antibody levels against several specific epitopes help in patient management rather than in the diagnosis. Using a modified Mab TB72 (anti 38 kDa antigen) competition assay, rapid detection of 50% of smear negative pulmonary and extrapulmonary cases were detected (Wilkins and Ivanyi, 1991). Inspite of all these, about 30% of the sputum positive cases remain negative by serology. Hence, it is clear at this stage that serology cannot replace old method of sputum microscopy. A combination of several tests should be evaluated considering the individual variation in antibody responses (Verbon, 1990). Inspite of the failures as mentioned above there is an immediate need for development of a cost-effective immunodiagnostic test for detection of tuberculosis which would immensely help the public health programme.

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IMMUNOPATHOLOGY OF TUBERCULOSIS

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In 1990, about 1,700 million people (one third of the world's population) were considered infected with *Mycobacterium tuberculosis*. In the same year, approximately 7.5 million cases occurred worldwide (95% of these from developing countries) leading to 2.5 million deaths (1). Another estimate indicates that lifetime risk of developing clinically evident tuberculosis following infection is only about 10% (2). These data bear testimony to the fact that a vast majority is able to mount an effective resistance or immunity against the disease. In the minority which succumbs, pathogenic mechanisms emanating from ever so labile host-parasite relationship overpower the protective mechanism. High virulence of pathogen together with low immunity of the host (e.g., in infants or HIV infected adults) significantly enhance the risk for this disease, which remains mostly pulmonary (~85% cases) in infected adults who are not 'generally' immunosuppressed.

1. Natural Course of infection

Fewer than 10% of the inhaled bacilli, in the shape of droplet nuclei containing 1-3 bacilli, can reach the alveolar spaces (3). Following a breach in the alveolar epithelium, they are phagocytosed by alveolar macrophages which remain in an 'activated' stage due to continuous exposure with nonspecific stimuli. The infection may get cleared or established, depending on the virulence and load of viable bacilli (at least 10-50 droplet nuclei may be needed to establish the infection) and immune status of the host. If established, the bacilli multiply and rupture alveolar macrophages. The released bacilli parasitise immature monocytes coming from blood and continue to multiply locally. Since antigen specific cell mediated immunity (CMI) has not yet developed, this state of 'symbiosis' continues for some time. After 2-3 weeks, the host becomes tuberculin positive and bacterial multiplication is arrested by initial caseous necrosis of the foci. Parasitised immature macrophages are destroyed by 'tissue damaging' immune mechanisms and microscopic caseous lesions occur. Bacilli can persist (without multiplication) in the solid caseous matter. Given the infectious load and host immunity, the lesions may either regress and heal or progress to produce grossly visible tubercles (caseous lesions/granuloma). From this point, the infection may adopt either of the following courses : (a) A strong CMI leads to arrest and clearance of infection, frequently for life. Clonal expansion of antigen specific T cells produces macrophage activating cytokines/factors and activated macrophages phagocytose and destroy bacilli escaping from the edge of the granuloma. The granuloma becomes walled-off and caseous centre inspissates. (b) If CMI is weak, bacilli escaping from the granuloma parasitise incompetent macrophages in the mantle. The granuloma gets enlarged as the host continues to use tissue damaging immune response. Local lung tissue is detroyed and

hematogenous spread may lead to miliary tuberculosis and eventually death. 'Cavity' tuberculosis, intriguingly, occurs in apparently immunocompetent (tuberculin positive) hosts; perhaps due to a subtle variation in tissue damaging immune response.

The caseous centre liquefies and bacilli multiply extracellularly and profusely in this fluid which serves like a culture medium. The cavity gets surrounded by a wall of collagen and macrophages, though activated, are unable to control bacterial multiplication or get killed in the process by the toxic bacterial products. Bacilli can spread through the airways to other parts of the lung and to the outside environment in the shape of infective droplet nuclei. Cavities may expand, shrink or heal over time and bacilli may persist in a healed cavity. Secondary lesions may be caused by hematogenous spread, reactivation of dormant bacilli or reinfection in small doses. In an immunocompetent host, activated macrophages converge rapidly at the secondary site and clear the infection.

2. Immune - mechanisms of protection

2.1 Uptake of *M.tuberculosis* and microbicidal action of macrophages

'Opsonised' or non-opsonised bacilli may enter the macrophage via specific binding to distinct surface molecules (receptors). The macrophage mannose receptor facilitates non-opsonic phagocytosis by recognising terminal mannose residues on target particles. Thus, phagocytosis of virulent strains of *M.tuberculosis* in the absence of serum can be inhibited substantially by soluble mannan, mannose albumin and anti-mannose capped liporarabinomannan (ManLAM) since treatment with anti-ManLAM monoclonal antibodies reduced *M.tuberculosis* binding to macrophages by as much as 49%. Binding of attenuated H37Ra strain was not blocked by these agents (4). Nonetheless, mannose dependent binding is likely to account for only a portion of all phagocytosed bacilli. Uptake of *M.tuberculosis* is also reduced in serum depleted of the third complement component (C3). Anti-C3 monoclonal antibodies were reported to inhibit monocyte adherence of preopsonised bacilli by 71%. Opsonisation with C3 could result from complement activation (through alternative pathway) by certain mycobacterial cell wall components, such as Man-LAM, leading to entry through the complement receptor CR3(5). A strain dependent non-opsonic binding of *M.tuberculosis* to CR3, mediated by 'capsular polysaccharides', has also been described (6). Further, association of pathogenic mycobacteria with complement cleavage product C2a result in the formation of C3 convertase which in turn cleaves C3 resulting in opsonisation with C3b. The mycobacteria opsonised in this manner are recognised mainly by CR195). The contribution of macrophage Fc receptors in phagocytosis of M.tuberculosis (following opsonisation with antibodies) remains uncertain. Because engagement with Fc receptors initiates a potent and microbicidal 'respiratory burst' within the phagocyte, successful pathogens are unlikely to use this pathway.

Having gained entry into macrophages, *M.tuberculosis* get confined within 'tight' vacuoles (phagosomes). The observation that some organisms may escape from these

phagosomes into cytosol (7) has not been substantiated by more recent studies (8,9). Phagolysosomal fusion, a highly regulated event, is a significant antimicrobial mechanism of phagocytes. For optimal activity of lysosomal digestive enzymes, milieu within the phagolysosomes is mainted acidic (pH~5) by ATP dependent proton pump (10). Activated macrophages also undergo a respiratory burst, generating 'reactive oxygen' and 'reactive nitrogen' intermediates (ROIs and RNIs) which enhance their microbicidal power. ROI-dependent restriction of mycobacterial growth is primarily mediated by H_2O_2 whose production is variable, with virulent strains inducing low levels of tumor necrosis factor (TNFα) which in turn governs the ROI levels (11). The mycobacterial cell wall constituent LAM has been found to trigger TNFα synthesis in macrophages(12). Singlet oxygen (O), hydroxyl radicals (OH) and hypochlorous acid (HOCL) are apparently not involved in killing (11). RNIs (nitric oxide, NO) can kill.M.tuberculosis under less harsh, more physiological conditions. Their inhibitory effect ranges from partial to negligible depending on the strain (13). Superoxide anion (O_2-, a ROI species) and NO can combine to transiently produce peroxynitrite (ONOO-) which is considered more cytotoxic than either of the constitutents (14). Increased production of inducible nitric oxide synthase (iNOS) and peroxynitrite has been shown in BCG inoculated human alveolar macrophages in vitro (15).

2.2 Mediators of macrophage activation

The capabilities of resident alveolar macrophages and recruited monocytes to destroy *M.tuberculosis* differ significantly and progressively through the course of infection. Initial infection leads to activation of alveolar macrophages through induction of cytokines which also serve to recruit additional leukocytes from peripheral circulation. Polymorphonuclear granulocytes (PNGs), residing in the vicinity of alveolar macrophages, might contribute to production in the early 'non-immune' phase. These cells are activated by *M.tuberculosis* (LAM) to produce chemokines interleukin-8 (IL8) and GROα. The former upregulates complement receptors CR1 and CR3 (for enhanced phagocytosis) and is also a chemotactic agent for T cells. GROα activates Natural Killer (NK) cells to produce interferon (IFN), a macrophage activator (16).

While alveolar macrophages may kill *M.tuberculosis* more effectively, immature monocytes recruited from periphery are thought to be less effective and hence serve as the organism's preferred host. Initiation of antigen specific T cell is essential for eradication of *M.tuberculosis*. The macrophage derived cytokine IL12 drives differentiation of naive T cells, towards helper-1 (Th1) phenotype. The act of phagocytosis itself is sufficient to trigger production of IL12, though coexistence of NK cells has been considered essential for its induction(17). Th1 cells, upon activation with appropriate mycobacterial antigens (proteins/peptides) displayed 'MHC-bound' on the surface of 'antigen presenting cell' (APC, e.g., macrophage), secrete macrophage activating lymphokines IFN and TNFα.While the former alone is capable of inducing a respiratory burst generating potentially cytotoxic ROIs, the generation of RNIs requires both (11,15).

2.3 Role of T cell subsets

T cells are obligatory mediators of protection. 'αß' receptor bearing T cells (of CD4+ or CD8+ categories constitute>95% of post-thymic T cells whereas γδ T cells (CD4-, CD8-; some CD8+) are a minority (<5%), though displayed prominently on mucosal tissues e.g., lungs. All T cell populations contribute to protection, perhaps in a stage or a time dependent manner. CD4+ T cells of Th1 specificity are the main source of IFNτ - a key activator of antimycobacterial immunity through a variety of macrophage stimulating machanisms, e.g., production of monokines IL1 and IL12, enhancement of expression of antigen presenting MHC class II molecules and antigen presentation, and generation of ROIs and RNIs. Recently, treatment of 'multi-drug resistant' tuberculosis patients with aerosolized IFNτ was found to produce very encouraging results (18). The cytolytic CD4+/ CD8+ T cells have also been considered essential for resolving the infection by promoting caseous necrosis which is considered as the hallmark of immune competence against *M.tuberculosis*. It is assumed that these cells lyse immature macrophages (especially when immunity is still to develop fully) to release viable bacillli which can be taken up and digested by mature and activated macrophages. However, an essential role for these cytolytic T cells has been disputed by the observation that deletion of perforin and granzyme genes (both essential for lytic pathway) does not influence the course of infection in mice (19). Apoptosis (Programmed cell death) of macrophages could be considered as an alternative mechanism for caseation. Extensive apoptosis, coinciding with local TNFα synthesis, has been seen within caseating granuloma in lung tissues from tuberculosis cases (20).The role of cytolytic γδ T cells suggested by indirect evidence. Their expansion primarily caused by low molecular weight non-proteinaceous components acting like superantigens, though mycobacterial proteins can also stimulate them (21).

3. Immuno-pathogenesis

3.1. Parasitisaton of host cells

It is suggested that the bacilli may gain access to alveolar macrophages by breaching the intercellular junctions of the alveloar epithelium. TNF α, produced by the epithelial cells in response to 'non-proteinaceous' components of *M.tuberculosis*, may downregulate bioelectric barrier properties of the epithelium to facilitate invasion in this manner (22). Since entry through Fc receptors may trigger a potent respiratory burst, virulent bacilli may prefer other, safer routes (e.g., through complement/mannose receptors) for parasitisation of the macrophages. Intracellularly, products of *M.tuberculosis* are known to inhibit phagolysosmal fusion as well as vesicle acidification. Vesicles containing viable M.tuberculosis appear to bud from the phagosomes with no subsequent fusion with lysosomes (7). This restricted capacity of mycobacterial phagosomes to fhese suggests that their biochemical composition is altered. Importantly, *M.tuberculosis* appears to prevent the phagosomes from developing into acidic compartments. Vacular membranes surrounding the bacilli lack proton ATPase, which may be required for phagosomal

acidification (10). Abundant production of ammonia by the bacilli could also play an important role in this respect. Ammoninum chloride affects saltatory movement of lysosomes and alkalinises the intralysosomal compartment (23). Several mycoacterial products including phenolic glycolipid-I, sulfatides and LAM can scavenge ROI or inhibit the respiratory burst that generates them. LAM also inhibits transcription of IFNτ inducible genes. ManLAM, which is a less potent inducer of TNFα production than AraLAM (12), may downregulate RNI production. Macrophage derived cytokines IL10 and transforming growth factor (TGFß1) have also been reported to attenuate NO production and RNI mediated antimicrobial function (24,25). IL10 negatively regulates macrophage functions and promotes disease exacerbation by overriding signals delivered by IFNγ. Macrophage derived IL6 has also been reported to promote intracellular mycobacterial growth (26).

3.2. Modulation of T cell responses

IL10,IL4, and certain cell surface components including MhLAM may suppress IL12 production resulting in poor induction of the protective TH1 pathway (24,27). Further, activation of antigen specific Th1 cells may remain subdued due to containment of viable bacilli within tight phagosomes resisting the degradative mechanisms. This may reduce the capacity of macrophages to process *M.tuberculosis* antigens and present them on MHC (class II/I) proteins to the specific T cells. IL6 also suppresses T cell activation (26). Finally, IL10 promotes differentiation of T cells towards Th2 pathway which is characterised by the synthesis of the cytokines associated with disease exacerbation and/ or immunosuppression (IL4, IL6 and IL10). IFNτ, on the other hand, downregulates the Th2 pathway. TH1 to TH2 switchover may be seen as a strategy to protect host against extensive tissue damage which could be caused by an unabated TH1 response vis-a-vis progressive infection.

4. Conclusion

Despite some impressive progress made in the recent past, our understanding of the immunopathology of tuberculosis is far from complete. Quantifiable host/parasite factors responsible for pathogensis through various stages of the disease (following the onset) need to be identified and weighed for their relative contribution. The enigma of 'anti-self' (tissue damaging) vs 'anti-parasite' immune responses needs to be resolved in terms of (a) The host cells and *M.tuberculosis* antigens involved in these seemingly disparate mechanisms of parasitisation of host cells and evasion of intracellular killing. (b) Mechanism for caseous necrosis (c) How the ratio of tissue damaging and anti-microbial immune responses is governed and which qualitative/quantitative changes in them lead to cavity tuberculosis, and (d) What triggers a switch from immunogenic Th1 to pathogenic Th2 type of responses. Answers to these vital questions would pave the way for effective management of this disease which is being seen as a major threat to the mankind.

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SECRETORY ANTIGENS OF *M. TUBERCULOSIS*, THEIR DETECTION AND VARIATIONS WITHIN INDIAN ISOLATES

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Even though tuberculosis kills 3 to 4 million persons each year and half of the Indian population is estimated to be infected with tuberculosis, yet we remain rather ignorant about the nature of contemporary *M.tuberculosis* in our country. Most research has been carried out on standard *M.tuberculosis* strains, like H37Ra, H37Rv and Erdman strains which were isolated a century ago! Genetic drift in *M.tuberculosis* coupled with the genetic changes forced by the use of anti-tuberculosis drugs, would ensure that the strains causing infections now would be significantly different from the ones isolated almost a century ago. We have been engaged in studying the current Indian strains of *M tuberculosis*, specially to understand the variations in secretory antigens released by these strains. The idea is to look for shared antigens which could be utilized for vaccination and immuno-diagnostics purposes. We have about 50 current clinical isolates of *M.tuberculosis*. Parameters under study for the selected clinical isolates of *M.tuberculosis* are listed below:

- Basic data of drug sensitivity
- Adapt the isolate to growth in liquid media and record growth rates.
- Count CFUs and freeze sufficient bacteria in aliquoted suspensions.
- Isolate DNA and do comparative RFLP studies.
- Assess virulence of the strains in the mouse and guinea pig model*
- Collect secretory antigens (Cell free antigens or CFA) and compare their 2D gel patterns.
- Compare the immunological responses to CFA in mouse and human (*in vitro* systems)*
- Assess immunological response to isolates in mouse and human system*

(* We are restricted in conducting some of these studies at present, since we do not have biological containment facilities for keeping animals experimentally infected with *M. tuberculosis*).

Handling of Clinical Isolates of *M.tuberculosis*

The isolates are obtained on LJ slants, and in order to check potential contamination and loss of viability, they are transferred to fresh LJ slants, their growth monitored and drug sensitivity pattern tested. Clinical isolates are then transferred to small volumes of Sautons culture medium. Once the isolate starts growing in liquid medium, we gradually increase the culture volume so that sufficient bacteria are available for further work. Initially the isolates are grown in absence of Tween, in which case the growth is generally in form of bacterial clumps floating on the medium surface though for some strains, bacterial growth is also seen on the bottoms of the culture flasks. These cells are used for DNA isolation and culture supernatants are used as a source of secretory antigens. At a later stage, cultures are continued in medium containing 0.1% Tween, in which case the bacteria grow more homogeneously without forming significant clumps. These cultures are utilized for counting of bacteria by plating dilute culture suspensions on 7H11 agar plates and colony forming units (CFU) counted. Most strains of *M.tuberculosis* grow rather slowly and it takes 3 to 6 weeks for colonies to grow. Bacterial suspensions are quantitated by counting CFUs, large aliquots of suspensions are frozen for subsequent use in virulence and other studies.

Genetic Typing of Clinical Isolates

It was considered important to do genetic fingerprinting of the isolates so as to be sure of their being distinct. We have isolated DNA from several *M. Tuberculosis* clinical isolates, and attempted to do RFLPs by using a DR probe having the following sequence "GTT TCC GTC CCC TCT CGG GGT TCT GAC GAC". Using this technique, none of the Indian isolates of *M. tuberculosis* seem to be identical.

EcoRI digested DNA of some strains gave a band of about 1 kb size, visible over the normal smear on agarose gels. DNA was extracted from this band and was cloned in the EcoRI site of the pStuI (blue script) vector. Plasmids were isolated from 26 transformed white colonies and were further analyzed for the size of inserted sequences. Minor variations in insert sizes were observed which ranged from 0.8 to 1.2 kb. Three representative inserts (clone 6, 0.8 kb; clone 9, 0.9 kb; and clone 14, 1.1 kb) were selected and used as probes to assess cross hybridization with other inserts. Each insert cross hybridized with 3 to 4 other inserts indicating that these sequences could be repeat sequences. All three inserts were sequenced by using T3 and M13/pUC universal primers. Comparison of sequences with the available data base of DNA sequences indicated that clone 6 had an insert which was 100% homologous to a known sequence (Z80434 sequence and AD00012 cosmid of M. tuberculosis). Sequences determined for inserts from clones 9 and 14 however appeared to be unique though these had small stretches of homologies (60 to 75%) to a variety of sequences in the data base. Clone 9 and 14 were used as probes to assess there use in RFLP with several *M. tuberculosis* strains. These probes detected polymorphism which was less than the polymorphism seen with DR probe.

Virulence

In order to assess the virulence of the isolates, we have initiated animal infection model. C57B1/6 mice were given (i.v.) 10^6 *M. tuberculosis*. Different organs were homogenized at different time points and plated on 7H11 agar for counting CFUs. While we have standardized the procedures and protocols for these studies, work along these lines is currently stopped as we do not have biological containment facilities to keep experimentally infected mice.

Secretory Antigens

As the mycobacteria grow in liquid medium, they release secretory antigens (Cell free antigens or CFA) in culture medium. If the bacteria are allowed to grow to higher than a particular density, autolysis of the bacteria sets in and structural antigens are also released in the medium. We generally harvest the cultures at less than 10 days after seeding, in order to avoid structural antigens in the medium. We would like to make quantitative and qualitative comparisons of CFA derived from different Indian strains and the established strains like H37Rv etc, by using 2D gel electrophoresis technique. The concentration of CFA in culture supernatants are extremely low (range 5-15mcg/ml). Since about 25 mcg of CFA must be loaded on 2D gels, and one can load only 10µl of the protein solution, the protein from culture supernatants has to be concentrated about 200 fold. We have attempted a wide variety of concentration procedures like Amicon membrane concentration, Centricons, Ammonium Sulfate precipitation, acetone precipitation and lyophilization. We find that the 2D gel pattern changes with the technique used to isolate the antigens. The problem is furthermore compounded by the interference caused by other entities like LAM present in the culture supernatants. We already have CFA preparations from about 20 isolates and initial comparisons have interestingly shown significant differences in the 2D gel patterns of CFA derived from different isolates. One however has to be very careful in making sure that the observed differences are real and not artifactual. We have also attempted to do reverse phase HPLC separations of secretory antigen preparations and found interesting differences amongst strains.

Immunological Studies with Clinical Isolates and the Secretory Antigens

One of the aims of this study is to assess the immune response induced by different mycobacterial strains with the hope that the ones which are significantly potent in this respect may provide candidate antigens for a possible vaccine. Studies to assess the immune response in the mouse model, have been initiated. Using T cell proliferation assays, it appears that some of the secretory antigen preparations are more potent than PPD or sonicate antigen preparations.

Detection of Secretory Antigens, Potential as Immunodiagnostic Test

We have previously made detailed studies on the possibility of using *M.tuberculosis* antibody *and M. tuberculosis* antigens in serum and circulating immune complexes, but these parameters were not found to be useful as diagnostic tool for tuberculosis specially in a country like India where exposure of normal population to M. tuberculosis is high. We have recently developed a capture ELISA system to detect secretory (or cross-reactive) antigens which can detect even 1ng of antigen. Using this method, we could detect *M. tuberculosis* antigens in urine samples from confirmed tuberculosis cases. This procedure appears to have promise as an immunodiagnostic test for tuberculosis.

Collaborators and Co-workers

Prof. Ian Orme, (Colorado State University), Drs. P.K. Yadava and P.C. Rath (SLS, JNU), collaborated in different aspects of this work. Drs. Udaykumar, Anila Prabhu, Apurva Sarin, Ramesh Yadava, Anil Chander, Ms. Vidita Mallik, Ms. Saubiya M. Siddiqui and Mr. Shamik Ghosh have contributed to the work in capacities as Ph.D. students or post-doc workers. Mr. Zaved Siddiqui is the expert technician on this project.

Acknowledgment

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FATE OF INTRACELLULAR MYCOBACTERIUM TUBERCULOSIS IN ACTIVATED HUMAN MACROPHAGES

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Mycobacterium tuberculosis is a facultative intracellular pathogen that lives and multiplies in monocytes/macrophages.(1) It is believed that cell-mediated immunity and the associated ability of macrophages to destroy or inhibit the growth of the bacilli is all that is required to control infection with M. tuberculosis.(2) Before the tubercle bacilli can be destroyed by macrophages, these cells must be activated by T lymphocytes and/or their lymphokines. the subtype of T lymphocytes that are involved in inducing protective immunity against M. tuberculosis. the mycobacterial antigen(s) that are required to stimulate the protective T lymphocytes, the combination of cytokines and other factors that stimulate the macrophages which finally kill the mycobacteriaare still not known. Development of an in vitro model to study the relevant factors could yield valuable information in the understanding of of immunity to tuberculosis and also help in evolving immunomodulating agents as adjuncts to chemotherapy.

Several methods have been reported to study the macrophage microbicidal mechanism. Most attempts to assay stasis or killing of mycobacteria by macrophage. involve direct counting (3) (enumeration of bacterial counts by microscopy) or enumeration of viable bacilli released from disrupted cells. (4,5) Enumeration of colony-forming units (CFUs) is low and takes at least 6 weeks. Yet another method involves fixation, staining of the macrophage monolayers and the bacilli and counting the bacilli microscopically. Microscopic ounting of bacilli is difficult and time consuming. Tritiated thymidine incorporation into the released mycobacteria has not been universally accepted for use to assess stasis or killing of intracellular organisms. Incorporation of tritiated uracil into surviving organisms released form human monocyte layers has also been used (6) but this is open to criticism that one does not know whether the activity of the organism is altered or whether there is actually any change in the number of organisms. Our in vitro experiments were designed to answer the following questions: (i) whether normal monocytes from healthy individuals can kill or arrest the growth of tubercle bacilli and (ii) whether monocytes require activation for mycobactricidal function and if so what cytokines or factors are required? Enumeration of colony-forming units to assess the viability of intracellular organisms was carried out and the method is as follows:

Separation of mononuclear cells

blood (healthy volunteers)
⇩ ficoll hypaque density gradient
⇩ centrifugation

Separation of mononuclear cells
⇓ washed 3 times in HBSS
viability of mononuclear cells (by trypan dye exclusion)

Separation of monocytes

1 x 10^6 mononuclear cells/well in 96 well flat bottom plate
⇓ incubated for 45 minutes at 5%
⇓ Co_2, 37°C, humidified atmosphere
non-adherent cells removed by gentle washing
⇓
adherent monocytes were cultured in RPMI containing 5% autologous serum

Mycobactericidal assay
adherent monocytes either untreated or cytokine-treated were infected with *M.tuberculosis*
⇓
monocyte: M. tuberculosis (1:10)
⇓ 1 hour after infection extracellular
⇓ organisms were removed by gentle washing
infected cultures were maintained in medium alone or in medium containing cytokines
⇓
infected cultures were lysed at 1 hour, 24 hours, 46 hours, 72 hours and 1 week after infection
⇓
infected cultures were lysed at 0.8% digitonin
⇓
lysate was diluted with distilled water
⇓
diluted lysate was plated onto 7H with OADC to observe mycobacterial colonies.

The result of our experiment is represented in the following figure. Figure 1 shows that normal human monocytes restrict the growth of M. tuberculosis for 72 hours. after which the bacilli multiplied inside the intracellur environment. It is well known that monocytes can be activated by LPS, PMA and recombinant cytokines like TNFα and IFNγ. NOrmal human monocytes cultured in vitro and stimulated with LPS released TNFα and its production can be enhanced by IFNγ. Noth TNFα and IFNγ have been shown to enhance the microbicidal nature of macrophages.[5,7-13] Hence we chose IFNγ. TNFα and LPS as our activating agents. We observed that activation of human macrophages with non specifics stimulants like LPS and PMA, either alone or in combination with cytokines like recombinant IFNγ and TNFα failed to kill or arrest the growth of intracellular M. tuberculosis (figure 2)

Recently, Molloy et al[14] highlighted that control of tuberculous infection occurs in a granuloma which is intimately associated with the accumulation, activation and death of mononuclear leukocytes. they pointed out that cell death could be due to necrosis or apotosis and provided further data to show tat only apotosis (indiced by adenosine triphosphate [ATP]) but not necrosis, of chronically infected cells, resulted in 60 to 70% loss in the viability of intracellular bacilli Calmette-Guerin (BCG). They assumed that observations made on one mycobacterial species were relevant to the others. In our present experiments when M. tuberculosis - or M. Smegmatis-infected human monocytes were treated with ATP, a loss monocyte viability was observed confirming apotosis with no reduction in bacterial viability (Narayanan PR, unpublished data). Thus we were unable to demonstrate the stasis or killing of M. tuberculosis by activated human macrophages under in vitro conditions.

Figure 1. Fate of *M. tuberculosis* inside human monocytes *in vitro*.
Human monocytes (0.1 x 10^6 cells/well) were infected with *M. tuberculosis*. For every single monocyte, 10 bacilli were added and incubated at 37°C for 1 hour. Extracellular organisms were removed by gentle washing. The infected cultures were terminated at the following time points, 1, 24, 48, 72 hours and 1 week after infection.

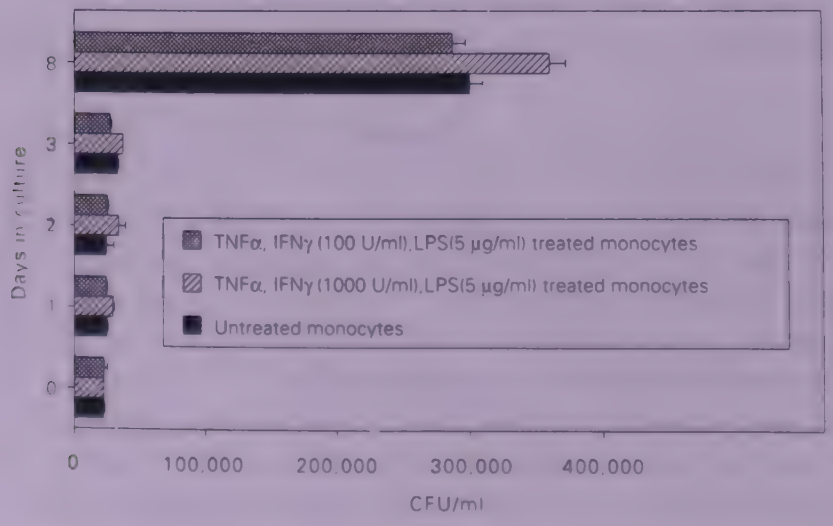

Figure 2. Fate of *M. tuberculosis* inside tumor necrosis factorα-, interferonγ- and LPS-treated human monocytes *in vitro*.
Human monocytes (0.1 x 10^6 cells/well)were treated with TNFα, IFNγ, at 2 different concentrations 1000 U/ml and 100U/ml. 24 hrs later, the cultures were infected with *M. tuberculosis*. Extracellular organisms were removed by gentle washing. The infected cultures were maintained in RPMI containing TNFα, IFNγ at 2 different concentrations, 1000 U/ml and 100 U/ml along with LPS at 5 mg/ml. The infected cultures were terminated at the following time points 1, 24, 48, 72 hours and 1 week after infection.

USE OF STREPTAVIDIN MAGNETIC BEADS IN SINGLE STRAND CONFORMATION POLYMORPHISM PROFILES TO DETECT MUTATIONS IN rpoB GENE OF M.TUBERCULOSIS.

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Introduction

Single strand conformation polymorphism (SSCP) is one of the promising techniques to identify mutations in short pieces of DNA (Orita *et al.* 1989). In this technique, DNA of interest is often amplified by the polymerase chain reaction (PCR) and then denatured by heat or alkali treatment before electrophoresis on a non denaturing polyacrylamide gel. Differences in mobility of either of the single strands compared to the control DNA indicate mutations which affect the secondary structure and alter the mobility of the DNA. We applied PCR-SSCP for the detection of mutations in the rifampicin resistance determining region (RRDR) of the *rpoB* gene of *M.tuberculosis* (Telenti *et al.* 1993a; 1993b). A nested PCR was used to amplify the RRDR. In the first PCR, 293-bp product was amplified and in the second PCR a 103-bp of the first PCR product was amplified. However, in our experience using denaturation by alkali or heating, the denatured PCR product most often reannealed to form a large proportion of double stranded DNA during the electrophoresis (Selvakumar *et al.* 1997a). After visualisation by staining with ethidium bromide or silver staining, most of the DNA was in the double stranded form, with very little or no single stranded DNA. The single strands that could be observed often ran close together, making analysis of any difference in mobility difficult. Therefore an attempt was made to generate biotinylated PCR product using a biotinylated forward primer and later the biotinylated strand was separated using sterptavidin magnetic beads. The separated strands eliminated the problem of strand reannealing during SSCP and were silver stained to detect the shift in the mobility. Since the nested PCR requires more time and is more expensive, a biotinylated PCR product was generated in a single PCR using a biotinylated forward primer and an unbiotinylated reverse primer. This simplified protocol was applied to clinical isolates in an attempt to detect rifampicin resistance (Selvakumar *et al.* 1997).

Materials and Method

Clinical isolates:

The clinical isolates were obtained from the pulmonary tuberculosis patients attending Tuberculosis Research Centre, Chennai. Fifteen rifampicin resistant isolates and 6 rifampicin sensitive isolates were selected and coded. One rifampicin sensitive clinical isolate of *M.tuberculosis* was used as reference.

Extraction of DNA:

The DNA was extracted from the cultures by the procedures described by Baess (1974) and was used as template. The PCR was carried out using 0.5 ml microcentrifuge tubes in a Hybaid Omni Geni thermo reactor.

Nested PCR:

First PCR: Two hundred and ninety three base pairs comprising of RRDR of the rpoB gene was amplified by a first PCR. The PCR mixture (20 ul) contained 50 mM Potassium chloride (KCl), 10 mM Tris-Hydrochloride (Tris - HCl; pH 8.3), 1.5 mM Magnesium chloride (MgCl), 5% Dimethyl sulfoxide (DMSO), 200 ul (each) of dATP, dGTP, dCTP and dTTP, 10 pM each of a forward- rpo B FO (5' **CGT TGA TCA ACA TCC GGC CGG TGG** 3') primer and a reverse- rpo BRO (**5' TTT CGA TGA ACC CGA ACG GGT TGA C** 3') primer and 1 unit of Taq polymerase (BioTaq). The reaction mixture was denatured at 93°C for 2 minutes followed by 35 cycles denaturation (93° C) , annealing (58°C) and extension (72°C) C for 1 minute each. The reaction was terminated after a final extension at 72°C for 10 minutes.

Second PCR:

One hundred and three base pairs of the first PCR product was amplified in the second PCR. The reaction mixture contained the same ingredients as above except for the biotinylated forward- rpo B FIBio (5' **GT TCT TCG GCA CCA GCC AG** 3') and unbiotinylated reverse- rpo B SRI (5' **CAG ACC GCC GGG C CC** 3') primers. The reaction mixture was denatured at 93°C for 2 minutes followed by 20 cycles of denaturation (93°C), annealing (52°C) and extension (72°C) for 30 seconds each. The reaction was terminated after a final extension at 72°C for 10 minutes.

PCR to generate 253-bp product

A 253-bp fragment comprising of RRDR was amplified by PCR. The PCR mixture (20 ul) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl, 5 percent DMSO, 20 uM each of dATP, dGTP, dCTP, dTTP), 10 pM each of a biotinylated forward- *rpoB* FI Bio (**5' GT TCT TCG GCA CCA GCC AG 3'**) and an

unbiotinylated reverse- *rpoB* RO (**5' TTT CGA TGA ACC CGA ACG GGT TGA C 3'**) primer (the primers were synthesised at R & D Systems Europe Ltd. UK) and 1 unit of Taq polymerase (Bio line). The reaction mixture was denatured at 93°C for 2 minutes followed by 35 cycles each of denaturation at 93°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. The reaction was terminated after a final extension at 72°C for 10 minutes. The products were checked on 2 percent agarose gel electrophoresis using the standard procedures.

Separation of DNA strands

1. Heat denaturation: To 10 ul of the nested PCR product, 5 ul of loading solution (0.25% bromophenol blue, 0.25% xylene cyanol in 95% formamide) was added and heated at 95°C for 5 minutes. The contents were cooled in ice for 3 minutes before loading immediately on to the gel.

2. Alkali denaturation: To 10 ul of the nested PCR product, 2 ul of 0.5 M NaOH-10 mM EDTA solution was added and heated at 45°C for 5 minutes. The contents were cooled and loaded as above.

3. Separation of biotinylated strand by streptavidin beads: The biotinylated PCR product was captured using streptavidin magnetic beads as per the instructions given by the manufacturer (Dynal UK). In brief, 40 ul of the beads, suspended in 2x binding and washing buffer (BW buffer; 10 mM Tris, 1 mM EDTA and 2 M NaCl), was mixed with 20 ul of PCR product and made upto 80 ul. The mixture was incubated at room temperature (RT) for 30 minutes. After washing with 1x BW buffer, the captured DNA was denatured by adding 8 ul of 0.1 M NaOH and incubating for 10 minutes at RT. The alkali containing the denatured unbiotinylated strand (UBS) was aspirated, made upto a volume of 50 ul in sterile distilled water and then precipitated by the standard ethanol-sodium acetate method. The precipitate was resuspended in 5 ul of 1x TE buffer. The beads with the captured biotinylated strand (BS), were treated with 50 ul of 0.1 M NaOH and washed a further 3 times with 1x BW buffer and finally suspended in 5 ul of 1X TE buffer. The separated strands were heated at 95°C for 5 minutes with an equal volume of loading buffer and then cooled and loaded as above.

Polyacrylamide gel electrophoresis:

A 10% gel was prepared by mixing 10.5 ml of 40% acrylamide-bisacrylamide (49:1; Sigma), 27.4 ml of deionized water, 2.1 ml of 10x TBE buffer, 2.0 ml of glycerol, 21 ul of TEMED and 210 ul of 10% ammonium persulphate (Sambrook *et al.* 1989). The dimensions of the gel were 180 x 160 x 1.6 mm. The electrophoresis was carried out in Hoefer SE 600 vertical unit using 0.5x TBE buffer at 200 volts for 3 hours at RT. The separated strands were visualised by silver staining.

Silver Staining:

The DNA strands in the gel were stained with silver as described by Ainsworth *et al.* (1991). In brief, the gel was treated with ethanol (10%), Nitric acid (1%) and silver nitrate (0.2%)-formaldehyde (0.1%) solution, respectively for 5, 3 and 20 minutes. After washing 3 times with double distilled water the gel was treated with sodium carbonate (3%)-formaldehyde (0.025%) solution to develop the stain. The gel was preserved in 10% glacial acetic acid and photographed.

Criteria for rifampicin resistance:

The slow or fast migration of either of the strands, compared to the corresponding strands from the rifampicin sensitive reference control, was considered as indicative of rifampicin resistance.

Results

Heat and alkali denaturation of PCR products for SSCP analysis proved unsuccessful in our hands for the generation of single stranded DNA products (Fig.). During SSCP analysis the single DNA strands were found to reanneal to yield double stranded product which was visualised after staining. On the other hand prior separation of the DNA strands using streptavidin magnetic beads consistently yielded good SSCP analysis. Treatment of DNA strand with S1 Nuclease prior to SSCP analysis confirmed the single-strand nature of the stained bands (results not shown).

The observation of the migration patterns of UBS and BS in 21 samples is given in the table. In 3 samples (No. 23, 38, 44) the migration of UBS was not determined either due to poor staining of little or loss of DNA. It can be noted that 13 of UBS and 11 of BS showed difference in their migration pattern compared to the corresponding control strands. Eleven of 18 UBS migrated slowly compared to 5 of 21 BS. Only 2 of 18 UBS migrated fast while 6 of 21 BS did so. In 7 samples the difference in migration pattern was exhibited by both UBS and BS and in another 7 samples it was by either of the strands. In this study, 14 of 15 rifampicin resistant strains and 3 of 6 rifampicin sensitive strains were correctly identified by the PCR-SSCP.

Discussion

We experienced rapid reannealing of heat or alkali denatured DNA which prevented the separation of single-stranded products upon SSCP analysis. Factors that affect SSCP analysis were discussed by Yap and McGee (1994). One of the factors, that is the reannealing of the strands, especially when the product size is less than 100-bp, reduces the efficiency of SSCP analysis. The reannealing of strands can take place while the samples are being loaded and during the initial period of electrophoresis before the DNA has entered the gel. Another factor could be due to large quantities of DNA (0.5 to 1.0 ug) applied to the gel for a strong signal after silver staining. A higher concentration of DNA is likely to promote rapid reannealing. Other detection methods, involving

radioactive labeling of PCR products, for example, may not encounter such a problem because less DNA is needed to generate a strong signal after SSCP. The use of radioactivity has its disadvantages, mostly in the hazardous nature of the use of radioisotopes. Yap and McGee (1994) had found that the alkali denaturation resulted in better separation of single strand DNA because the alkali prevents the reannealing of strands. They also stated that heating at 42°C for 10 minutes before loading on to the gel reduced reannealing on some occasions. Both these were tried in our samples but we failed to get better separation. In order to overcome the reannealing of strands, a novel approach was attempted. In this, a biotinylated primer was used in PCR. Then, the biotinylated and the unbiotinylated strands were separated and subjected to SSCP by PAGE. This approach also separates the two DNA strands in different lanes of the polyacrylamide gel, a process that is beneficial if the two strands have similar mobility that might confuse analysis. The recognition of shift in the migration of strands was thus made easy. In this study we proved an adaptation to the PCR-SSCP protocol which enables large amounts of DNA to be loaded onto the gel for subsequent silver stain detection and avoids the problem of single strand reannealing.

Another factor, that is the multiple conformations of strands would also limit the efficiency of PCR-SSCP analysis. The SSCP analysis of the PCR products of all our samples produced 4 bands in a different gel format and protocol (data not shown). This could be attributed to the possible two conformations of each of the strands while the other possibilities cannot be ruled out.

Similarly, Telenti *et al.* (1993a) observed a three-band pattern in his samples. In the present study, the occurrence of the multiple conformations of strands was not observed although it remains to be explained.

A large scale study to determine the association of the migration pattern of single stranded DNA with the specific nucleotide change in the *rpoB* gene might be useful for the presumptive identification of specific mutants in the clinical isolates. It is interesting to note that Telenti *et al.* (1993a) observed a specific migration pattern for each of the nucleotide substitution.

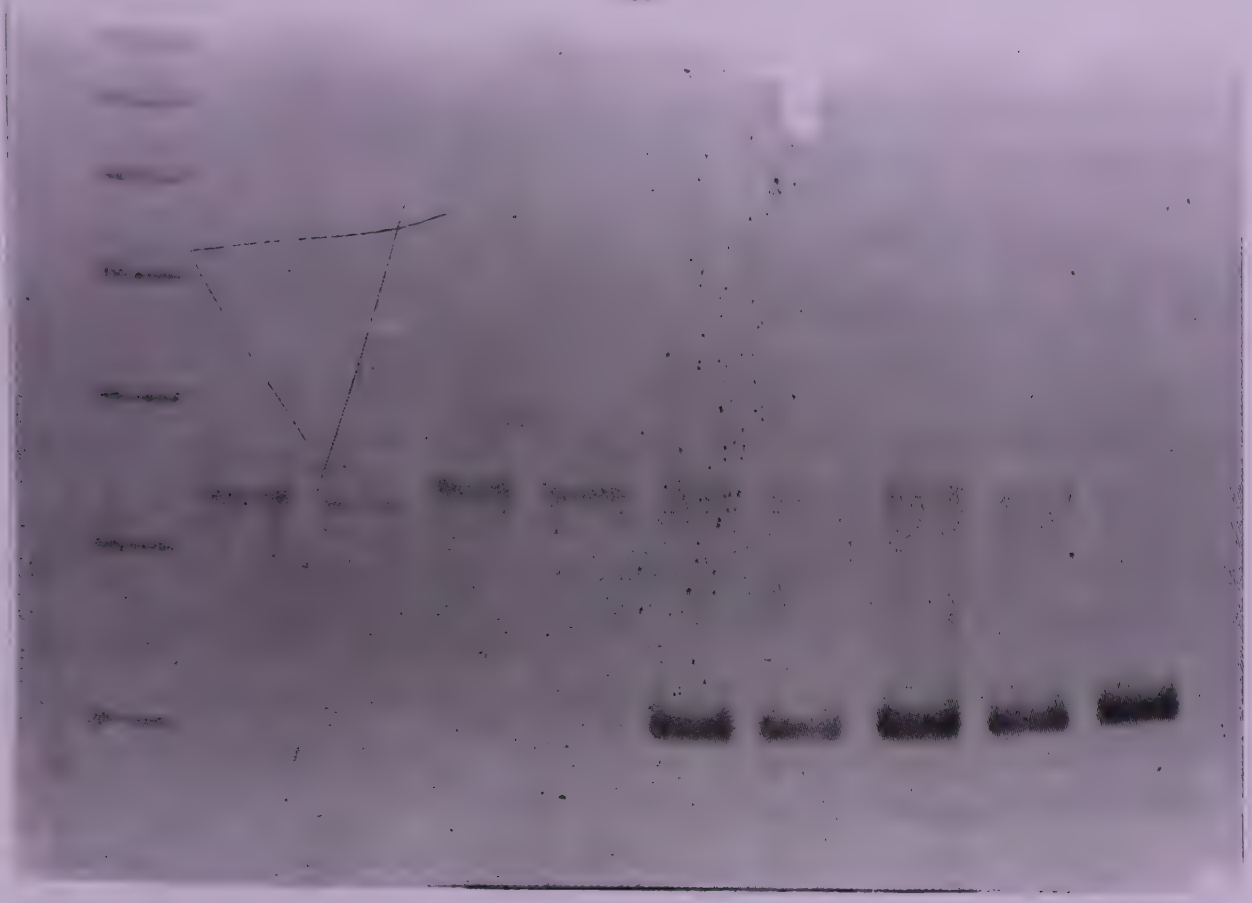
In this study 3 (No. 6, 20, 21) of 6 rifampicin sensitive strains were misclassified by the PCR-SSCP. It should be realised that SSCP does not differentiate rifampicin sensitive strains with functionally silent sequence changes. Therefore, DNA sequencing of the PCR products only could confirm the mutations occurring in these 3 specimens. Also, 1 (No.4) of 15 rifampicin resistant strains was misclassified by PCR-SSCP in the present study. On scrutiny, it was found that this isolate was obtained from a patient whose alternative isolates were sensitive to rifampicin. It should be pointed out that using different protocols such as automated PCR-SSCP (Telenti *et al.* 1993b) and automated sequencing (Kapur *et al.* 1994), variations in the classification were reported. In the former, 2 of 66 and in the latter 3 of 121 rifampicin resistant strains were misclassified.

The present PCR-SSCP format takes less time and is less expensive as it involves only one PCR. It is known that there are few mutations which could not be detected by any of the sophisticated molecular techniques. The results suggests that this procedure can be tried for the detection of such mutations in the RRDR of *M.tuberculosis*. In addition, a separate study using large number of sensitive and resistant strains need to be carried out to assess the validity of the method. Also, attempts should be made for the early detection of rifampicin resistant *M.tuberculosis* in sputum samples of pulmonary tuberculosis patients as it is a surrogate marker of multidrug resistant tuberculosis.

Table. The observations on the mobility of DNA strands and the results of the classification of rifampicin sensitivity in 21 samples.

Sample No.	Mobility of		Classification by	
	UBS*	BS	PCR-SSCP	BACTERIOLOGY
1.	slow	slow	R	R
2.	slow	slow	R	R
3.	slow	equal	R	R
4.	equal	equal	S	R
6.	slow	equal	R	S
7.	fast	fast	R	R
8.	equal	fast	R	R
9.	slow	slow	R	R
11.	equal	equal	S	S
12.	equal	equal	S	S
13.	equal	equal	S	S
19.	slow	equal	R	R
20.	slow	equal	R	S
21.	slow	equal	R	S
22.	slow	equal	R	R
23.	undetermined	fast	R	R
32.	fast	fast	R	R
33.	slow	slow	R	R
34.	slow	slow	R	R
36.	slow	slow	R	R
38.	undetermined	fast	R	R
44.	undetermined	fast	R	R

* UBS: Unbiotinylated strand; BS: Biotinylated strand;
R: Resistant; S: Sensitive



Legend for the figure.
Lane 1. DNA marker
2. Biotinylated rif. resistant
3. Biotinylated rif. sensitive
4. Unbiotinylated rif. resistant
5. Unbiotinylated rif. sensitive
6. Alkali denatured rif. resistant
7. Alkali denatured rif. sensitive
8. Heat denatured rif. resistant
9. Heat denatured rif. sensitive
10. Undenatured double stranded DNA (Control).

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IDENTIFICATION OF THE PROMOTER OF AMIDASE GENE FOR EXPRESSION OF USEFUL, MYCOBACTERIAL GENES

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The genetics of mycobacteria has lagged behind because of several reasons. Mycobacteria grow very slowly, their generation time ranging anywhere between 12-24 hrs. Mycobacteria are rather hydrophobic and tend to grow in clusters and there is difficulty in purifying individual cells for genetic analysis. Very few genetic markers have been found in mycobacteria because there is no known naturally occurring genetic exchange in mycobacteria. With the creation of genomic libraries of *M. tuberculosis* more than 50 genes have been characterised. Many of them are not expressed efficiently in *Escherichia coli* (E.coli) under the control of their own promoters, since very few mycobacterial promoters are recognised by the *E.coli* transcription machinery. This clearly shows that mycobacteria use a different system of gene regulation. Understanding the gene regulation of mycobacteria might throw light on the slow growth rate, about their persistence in a resting phase and also about their intracellular survival. Besides this if inducible or strong promoters are identified they can be used in over expression of genes coding for proteins useful in diagnosis and protection.

We chose to study the regulation of acetamidase gene of *M. smegmatis*. Acetamidase gene is the first inducible gene of mycobacteria identified by Halpern and Grossuies, purified to homogeneity and shown to be inducible by Draper. Eshwar *et al.*, has cloned and sequenced the acetamidase gene and 1.5 kb upstream sequence. A further 1.4 kb of upstream sequence has been determined by Tanya *et al.*,. They have indicated that the regulation is by positive and negative control and at the mRNA level. We wanted to further study the transcription start site and identify the actual promoter so that it can be used to express other mycobacterial genes.

We took a PCR cloning approach. We designed primers from the sequence of the upstream region of the amidase gene. The fragments were constructed by PCR technology. These were used as probes to identify the size of the amidase transcript - on a northern blot. The RNA was isolated using two different methods from induced and uninduced cultures of *M. smegmatis*. This RNA was found to be suitable for norther blot and primer extension analysis.

The norther blot revealed 2 products of 3.0 kb and 1.2 kb. These 2 bands were recognised by all four probes of the upstream amidase gene. These results suggests that the whole amidase transcript is 3.0 kb in size and is then processed further.

In order to identify the transcription start sites, primer extension analysis was carried out. Oligonucleotides (18 mers) complementary to the various regions upstream of the coding region were used in the primer extension reactions. The primers were designed to hybridise 200 bases apart since this provides optimal sensitivity in the primer extension reaction. Of 12 oligonucleotides used, 2 gave consistent products.

The two primer extension products were confirmed and the transcription start sites were deciphered by running sequencing ladder generated using the respective primers and the template. From these experiments it is clear that amidase gene is transcribed as a polycistronic message. A larger transcript of 3.0 kb is formed which is further processed at a single site to give amidase gene product of 1.2 kb and the other product coding for orfs 1,2,3. This promoter is currently being used to express important mycobacterial proteins.

The Modulation Of Immune Response By BCG Vaccination In South India

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INTRODUCTION

Tuberculosis with HIV and the resurgence of multi drug resistant strains of M.tuberculosis are the new challenges in the field of Tuberculosis. The only strategy for protection so far has been to enhance the antimycobacterial resistance of the host by vaccination with M.bovis BCG developed earlier, however and any improvement has not yet been achieved. The problem is more serious as the efficacy of BCG vaccination is now coming into question because the human trials world over have failed to demonstrate significant protection (1). The low level of protection by BCG vaccination in South India's Chingleput population has many explanations (2). The natural immunity raised against environmental mycobacteria is one of the explanations given for the failure of BCG vaccination in this population (3).

The protective immunity is a complex cascade of cellular interactions mediated by a network of cytokines. A correct combination of cytokine production and cellular activation will determine the successful protective response by modulating the immune response either towards Th1 or Th2 type.

We know that BCG vaccination has not given protection against tuberculosis in South India (2), however, it causes skin conversion from a Mantoux negative to a positive status. This shows that BCG does cause certain alterations in the T cell repertoire which in turn might alter the cytokine secretion pattern. Hence, in the present study, we tried to conduct a phenotypic functional analysis of T cells in PRE and POST BCG vaccinated subjects. We studied the cell mediated immune responses (CMI) in vitro by assessing the skin test convention, lymphocyte proliferation and the cytokine secretion patterns before and after BCG vaccination in Mantoux negative subjects and compared with that of PPD +ve subjects. BCG induced changes in these parameters will help us to assess whether BCG vaccination modulates the immune response towards protection in this population.

MATERIALS AND METHODS

Study Population : The subjects were selected from villages in the Chingleput district of Tamil Nadu adjacent to where the major BCG vaccine trial was carried out (2). Selection was initially on the basis of age and a Mantoux skin test considered negative by the criteria of the vaccine trials. The subjects were either male or female and their ages

ranged from 13 to 22 yrs (mean 16.5). Clearance by local medical ethics committees and informed consent were obtained. All subjects met stringent criteria of eligibility, which included good health; normal chest radiographs; and no history of tuberculosis or other chronic diseases, of anti tuberculosis chemotherapy, or of BCG vaccination.

Group I : Totally 20 subjects were chosen and were Mantoux skin tested with 1 TU of RT23 with tween 80 (King Institute, Guindy, Madras) and all were found Mantoux negative for the same (72 h reaction diameters, 0 to 7 mm to 1TU RT23). These subjects formed the period PRE group. Immediately before vaccination, a blood sample was collected and vaccination was done by Intradermal injection of 0.1 ml of the freeze-dried Danish BCG strain 1331 (King Institute, Madras). After 8 weeks of BCG vaccination, blood was again taken and the PPD-S skin test was repeated. These formed the POST group samples from the same individuals.

Group II : Forms 20 healthy tuberculin reactive (PPD +ve) control subjects from the same area.

Preparation of PBMC

30 ml of heparinised blood was collected from all the individuals and PBMC were separated on Ficoll-hypaque gradient. The cells were washed and suspended in RPMI 1640 (Sigma) culture medium supplemented with 10% heat inactivated human AB serum and used for the following assays.

T cell proliferative assay

The single cell suspension was adjusted to 1 x 10^6 cells/ml and 200 µl per well was dispensed into 96-well round bottomed tissue culture plates (Costar). The cultures were set in triplicate and stimulated with PHA (1 µg/ml) for 3 days and with antigens (PPD 10 µg/ml and Heat killed M.tb 10 µg/ml) for 5 days at 37°C in Co_2 incubator. The control wells had only cells. The cultures were pulsed with 1 µCi of [^{3}H] thymidine per well, 24 hours before termination and the thymidine incorporation was measured. The stimulation index (SI) was calculated. The S1 values of 3 and above was arbitrarily chosen as significant value.

Reverse Transcriptase - PCR (RT-PCR)

In vitro experiments were set up with different mitogens (PHA-1 µg/ml) and mycobacterial antigens (PPD-10 µg/ml and Heat killed M.tb - 10 µg/ml), based on previous standardisation experiments. 1 x 10^6 PBMC cells/ml/well were dispersed in duplicate in 24 well culture plate (LINBRO). The cells were stimulated with respective antigens/mitogens for 24 hrs, 48 hrs and 96 hrs. The control wells had cells alone. The supernatants were collected for all the time points and stored at -70°C for cytokine estimation by ELISA. The cells were washed and lysed with lysis buffer. The total RNA was extracted and mRNA transcripts were reverse transcribed into CDNA by the method of Ehler et al., (4). The primers for the T cell cytokines IFNγ, IL4 and IL10 were

prepared and initial experiments were carried out to optimise conditions for each set of primers. Amplifications of CDNA using specific cytokine primers by PCR was done and the results of these experiments were expressed in relation to expression of the house keeping gene b actin using a semiquantitative scoring systems.

ELISA for cytokine measurement in supernatants

The supernatants collected at different time points were used for estimating cytokine levels by ELISA. The T cell cytokines IFNg, IL4 and IL10 were estimated by using ELISA kits from Amersham. The results were expressed as pico gm of cytokines per ml of supernatants, based on the standards provided in the kits. The data are expressed as means and standard error of mean. Student's 't' test was used for testing the significance of difference. Significance was accepted if $P<0.05$.

RESULTS

Skin Test Conversion

The age, sex and skin conversion results of the vaccinated individuals and PPD +ve group are given in Table 1. Except one, all the 17 subjects from PPD -ve group became PPD +ve after 8 weeks of BCG vaccination, confirming the BCG vaccination does cause skin test convertion.

Lymphocyte Proliferative Responses

In order to evaluate the degree of T cell activation after BCG vaccination, T cell proliferative responses were measured in PRE and POST BCG vaccinated subjects as also in PPD +ve subjects which served as positive control group for these experiments All the subjects except one showed very high response (Stimulation Index (S1) from 4 to 830) with PHA, however with specific antigen PPD, the response was moderate (SI from 1-94) and further low with heat killed M.tuberculosis (SI from 1-47). With mycobacterial specific antigens i.e. PPD & M.tuberculosis, the PPD +ve subjects are clearly divided into low responders (SI<24) and high responders (SI>25), however when the analysis was done from PRE to POST, it was found that there was no change in SI after BCG vaccination, except in 3 individuals where SI has increased after BCG vaccination (data not shown).

Reverse Transcriptase-PCR (RT-PCR)

Initial experiments were carried out to optimise the assay conditions and time kinetics. Based on these preliminary results, it was decided to estimate the T cell cytokines, i.e. IFN$_\gamma$, IL4 and IL10 at 48 hr time point by RT-PCR. Totally 12 pairs of BCG vaccinated subjects were screened for IFN-$_\gamma$, IL4 and IL10 expression. Also 12 control subjects from the PPD positive group were screened for the same cytokines All the values were expressed in gradation based on bactin genes. In PRE BCG group (PPD -ve), nearly 75 to 80% have expressed IFN$_\gamma$, IL4 and IL10 cytokines upon PHA stimulations, however with PPD & M.tuberculosis the expression was only 40 to 65%. In

PPD +ve group, 40 to 60% expressed IFN_γ, IL4 and IL10 upon PHA stimulation, while with PPD and M.tuberculosis the expression was only in 15 to 50% range (data not shown).

As the RT-PCR results were semiquantitative and it was very difficult to ascertain the real changes in the cytokine levels after vaccination, we decided to do ELISA tests on all these samples. The RT-PCR results were then compared with the ELISA values and it was found that there was a general correlation between RT-PCR results and ELISA values.

Cytokine Assays by ELISA

The T cell cytokines IFN_γ, IL4 and IL10 were estimated by ELISA in BCG vaccinated subjects and PPD +ve control subjects. There was no significant difference in IFN_γ levels in any of these groups when stimulated with PHA, but with PPD and M.tuberculosis antigens, one or two subjects increase in IFN_γ levels after BCG vaccination (Fig.1).

However, when the IFN_γ levels were compared in PRE BCG group with PPD +ve group, the IFNg levels were significantly increased in PPD +ve group with PPD antigen (P,0.01) and Heat Killed M.tuberculosis antigen (P,0.05).

With PHA stimulation, all the subjects showed significant increase in IL4 levels. After vaccination ($P<0.0001$) also PPD +ve subjects showed increase in IL4 levels when compared to PRE BCG subjects (i.e. PPD -ve subjects) ($P<0.005$). With PPD and M.tuberculosis antigens, the IL4 levels were very low (<2 pg/ml) and there was no major difference between the groups (Fig.2).

All the subjects have shown high levels of IL10 secretation in control group without any stimulus. With PHA and mycobacterial antigens, IL10 values were not significantly altered in various groups (data not shown).

DISCUSSION

Mycobacterium bovis BCG, since its discovery and first application as a vaccine to prevent tuberculosis in 1921 (5) has been the subject of controversy. Different clinical trials have established its protective efficicacy to range from 0 to 80% (6). Many factors interfering with immune responses, such as contamination with environmental mycobacteria, genetic diversity of vaccinated populations, follow-up of tuberculosis cases in small children, and quality of BCG vaccine strains may also interfere with the efficacy of BCG vaccination to give such divergent results (7). However, in a recent meta-analysis of the published literature on the efficacy of BCG vaccine in the prevented of tuberculosis, it was found that the BCG vaccine not only prevented 80% of severe forms of tuberculosis but also gave 50% protection across many populations and many forms of tuberculosis(8).

It appears very difficult to estimate, the impact of BCG vaccination on protection from the results available from the few clinical comparative trials performed so far, in addition there is a need for good assay system to predict vaccine-derived protective immunity in the population. The simplest and most popularly used method so far is the post vaccinated tuberculin conversion test which is considered as a sign of an "effective" BCG vaccination in many animal models, however the data from human populations have shown no evidence of a relation between BCG-derived delayed-type hypersensitivity (DTH) and protection (9,10,11). Hence a good immunological assay to measure the protective immune response after BCG vaccination is needed. Here, in this study, we tried to measure the protective immune response after BCG vaccination by looking at the cytokine network especially IFNg, IL4 and IL10 representing the two arms (Th1 and Th2) of cellular immune response.

Inspite of many controversial reports on tuberculin conversion test, many still view it as a sign (if not an actual measure) of an 'effective' BCG vaccination. As a routine practice in any vaccination study we did the tuberculin skin conversion test in these subjects, and our results showed (Table 1) that except one, all the subjects became tuberculin-positive after eight weeks of BCG immunization. This shows that BCG had brought some alterations in the T cell repertoire and hence in immune response.

To assess and correlate the tuberculin skin conversion and actual immunological status after BCG vaccination, we studied the T cell proliferative response in pre and post-BCG subjects and in PPD +ve control subjects. Our data showed that all the subjects showed good proliferative responses to PHA and mycobacterial antigens like PPD and heat-killed M.tb. However, the proliferative responses did not change from pre to post (except in 3 individuals), thereby suggesting that BCG immunization had not affected the proliferative response much.

The skin test conversion results do not correlate or match with the proliferative responses in these groups and hence it cannot be looked as a marker for the 'effective' BCG vaccination or for T cell activation. On the similar line, the results of MRC trial, showed that with 'highly effective' tubercular vaccines, the degree of protection conferred on the individual is independent of the degree of tuberculin skin test sensitivity induced in that individual by the vaccination (9). In another study, Comstock reviewed the efficacy and tuberculin conversion data from all the BCG trials and concluded that 'the lack of correlation is obvious and underscores the futility of predicting potency from conversion rates (11).

To further assess the activating status of T cells, we studied the cytokines by RT PCR and ELISA. The semiquantitative results of RT-PCR on various T cell cytokines gave the over all picture of cytokine levels in these groups, however the meaningful conclusions were drawn with the ELISA results.

Cytokines such as IL-12, IFN$_\gamma$ and IL4 are known to direct the differentiation of naive T cells toward a Th1 or Th2 fate. IFN$_\gamma$ and IL12 skew toward Th1 development, while IL4 and IL10 are involved in Th2 development (12). Th1 responses appear to

mediate functions related to cytotoxicity and macrophage activation, and therefore, play an important role in combating intracellular microorganisms. On the other hand, Th2 responses are effective in helping B cells to produce antibodies and are important in combating extracellular bacteria and in the induction of humoral immunity.

In the study of lymphokines produced specifically by T cells responsible for the expression of protective immunity, Kawamura et al., had shown that IFN$_\gamma$ production may be an appropriate marker for CD4+ T cells protective against infection with M.bovis BCG (13) and listeria monocytogenes (14).

Our results on IFN$_\gamma$ levels in PRE and POST BCG vaccinated subjects showed that with PHA stimulation, IFN-γ levels did not changed in any of these groups. However with PPD and M.tuberculosis antigens, only one or two subjects showed an increase in IFN$_\gamma$ levels after BCG vaccination, thereby suggesting that BCG vaccination did not influence the IFN$_\gamma$ levels (Fig.1 a,b,c). However when the IFN$_\gamma$ levels were compared in PRE BCG (PPD -ve gr.) group with control PPD +ve group, the IFN$_\gamma$ levels were significantly increased in the PPD +ve control group, following stimulation with mycobacterial antigens thus reflecting a Th1 type of response (Fig.1 b & c). This suggests that the control PPD +ve subjects have already developed acquired protective immunity (API) or natural immunity against mycobacteria and this could be due to their maximal exposure to the environmental mycobacteria as per the popular belief and assumption in this area (3). In our previous reports, the tests with PPD-B confirmed that the South Indian vaccinees had a great sensitization to environmental mycobacteria of the M.avium, M.intracellular, M.scrofulaceum group than the British vaccinees (15). Furthermore, a 15 year follow-up of the BCG vaccination trial in Chingleput revealed a modest protective effect (17%) only in the youngest age group of vaccinees (1 to 14 years), comprising those sensitized least to environmental mycobacteria (16).

Interestingly there was no evidence of a Th1 response in the PPD-ve population (no increase in IFN$_\gamma$ levels) even following vaccination with BCG. This shows that BCG vaccination did not modulate the immune response in this population and hence no additional protection.

Many reports in animal models have shown the protective immunity imparted by immunization with mycobacteria (17-23) but very few reports are available on human subjects. In one study, which was conducted in the same BCG trial area of Chingleput, Cheng et al., (24) have reported that BCG vaccination did not enhance bacteriostasis with the Indians but did so with the Londoners. They also reported that vaccination had no effect on cytokine release from mononuclear cells - with either British or Indian subjects. In our results also, we did not find any increase in IFN$_\gamma$ levels, (which in turn activates the macrophages) after BCG vaccination in these subjects and thus supports the previous observation of Cheng et al., (24).

Recently Ravn et al, (25) have evaluated the BCG induced immunity in Danish subjects based on their prevaccination sensitivity to PPD. Their results showed that both previously sensitised and naive donors mounted basically the same Th1 type of response

but with the different kinetics. This study again emphasises the varied efficacy of BCG vaccine in different population.

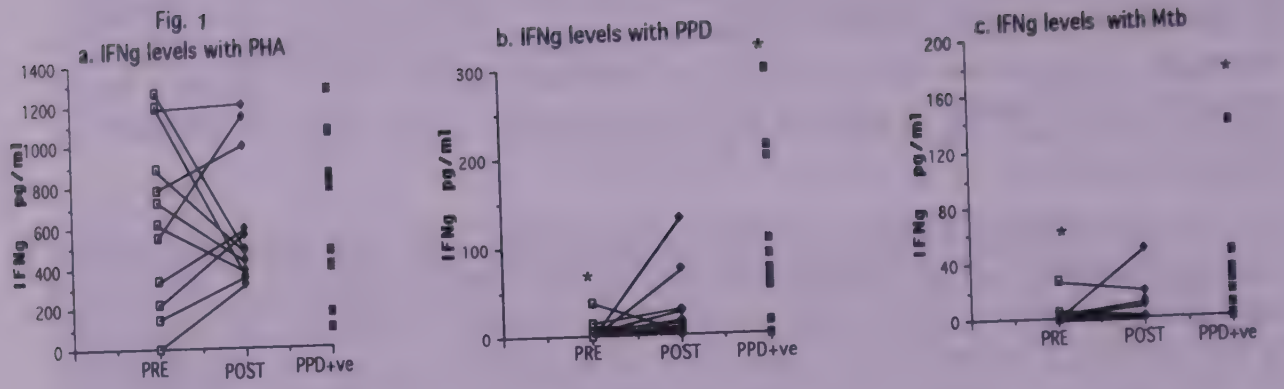
Taken all these reports together, IFN_{γ} seems to be the lymphokine distinctively required for the expression of protective immunity in humans, a broad spectrum of cytokines may also contribute to antimycobacterial immunity. Though very little is known about the distinctive features of protective T cells, they can be functionally characterised by the ability to produce IFN-g. However, it remains to be determined whether the difference of expression between DTH and protection against BCG is due only to the difference in the ability to produce IFN_{γ} by protective T cells and DTH-mediating T cells.

Our results on IL4 and IL10 levels in these groups showed no significant changes with mycobacterial specific antigens like PPD and heat killed M.tuberculosis. It has been suggested that under normal conditions the immune system a mechanism for suppression of IL4 production on stimulation with a Th1 antigen PPD(27), or the high concentration of IFN_{γ} and IL2 produced by PPD stimulation could be actively inhibiting IL4 production in normal individuals (28). Recently, it has been reported that in tuberculin-positive healthy subjects, in vitro stimulation with PPD preferentially induced IFN_{γ} and very few IL4 producing cells (29). Our results are in agreement with these reports and further show that BCG vaccination has not changed either IFN_{γ} levels or IL4 and IL10 levels, thereby suggesting that BCG vaccination in this population has failed to provoke a Th1 or Th2 response. On the other hand, increased levels of IFN_{γ} production following stimulation with mycobacterial antigens in the PPD +ve population reflects Th1 type of protective response.

Table 1

Skin test responses to tuberculin (1 TU TR 23) before (PRE) and 8 weeks after (POST) BCG vaccination.

	PRE	POST	PPD+VE
No.of Subjects	17	17	17
Age Range 13 - 22 (Mean)	16.5	16.5	18.6
Sex	11M/6F	11M/6F	10M/7F
BCG Scar Range (Mean)	0	6-9 mm 7.5 mm	not seen -
PPD Reaction size (Mean)	4.58 mm +/- 1.32	14 mm +/- 4.1	21.12 mm +/- 3.2



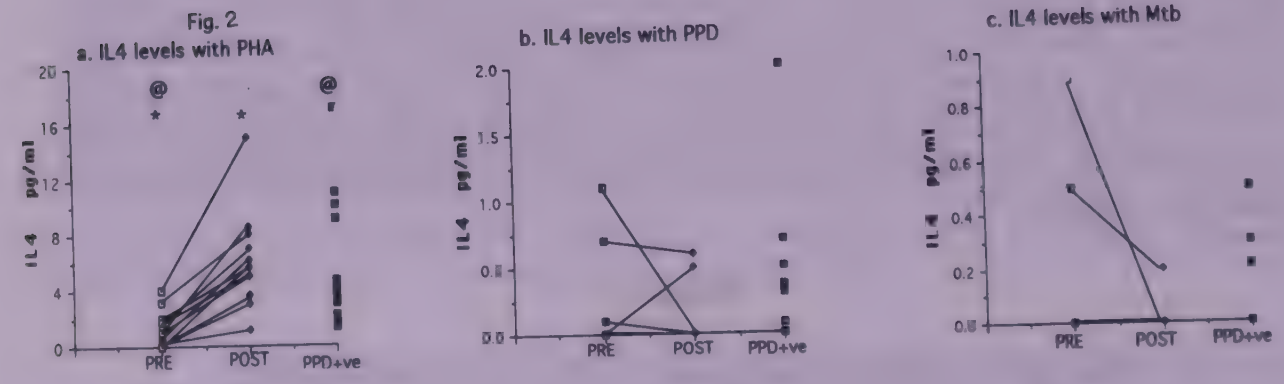


Fig.1 : IFN_γ levels in PRE and POST BCG vaccinated subjects and PPD +ve subjects. PBMC were stimulated in vitro with PHA (a), PPD (b) and Heat killed M.tuberculosis (c). IFN_γ levels were measured in 48 hours culture supernatants by ELISA and expressed as pg/ml concentration. Results represent individual values of 12 subjects in each group. Statistically significant differences ($^*P<0.05$, students 't' test) between PRE and PPD +ve groups.

Fig.2 : IL4 levels in PRE and POST BCG vaccinated subjects and PPD +ve subjects. PBMC were stimulated in vitro with PHA (a), PPD (b) and Heat killed M.tuberculosis (c). IFN_γ levels were measured in 48 hours culture supernatants by ELISA and expressed as pg/ml concentration. Results represent individual values of 12 subjects in each group. Statistically significant differences ($^*P<0.0001$) between PRE and POST groups and ($@P<0.005$) between PRE and PPD +ve group.

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NON-HLA GENE POLYMORPHISM IN PULMONARY TUBERCULOSIS

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INTRODUCTION

BCG vaccination has been shown to give protection against tuberculosis. However, South Indian (Chingleput) Trial of BCG vaccination did not give any protection against bacillary forms of tuberculosis. A number of hypotheses and possibilities were put forward for this failure (1). One of the possibilities suggested was the genetics of the people (Host genetics) living in that region. Pulmonary tuberculosis is a granulomatous lung disease caused by Mycobactrium tuberculosis. Susceptibility to tuberculosis has been suggested to be multifactorial. Though environmental and socio-economic factors are primarily related, numerous studies have emphasised the importance of host resistance and hereditary susceptibility (2,3).

The main objective of this project [**Part of the Indo-UK DFID (Department For International Development)** funded project] was to investigate the host molecular genetics in order to understand the failure of BCG vaccination as well as better management of tuberculosis.

The host genetics factors may be divided into HLA (Human Leucocyte Antigen) and Non-HLA genes.

HLA-studies in pulmonary - TB

HLA-studies carried out in Indian, Indonesian and Russian pulmonary tuberculosis patients revealed the HLA-DR2 association with pulmonary tuberculosis. Our recent study (4) also confirms HLA-DR2 association with PTB. Though HLA-DR2 association with PTB is significant ($P<0.001$; Relative Risk 2.3) (Table) it is only a minor association, which cannot be used as a genetic marker to predispose the disease, (e.g. HLA-B27 is being used as a HLA-genetic marker for ankylosing spondilytis). This suggests that association of Non-HLA genes with pulmonary tuberculosis may also be possible. Recently, association of multicandidate genes including HLA and Non-HLA genes have been suggested for various infectious disease (5).

Non-HLA gene polymorphism

Human genome analysis revealed several candidate Non-HLA genes. Due to point mutations, most of these Non-HLA genes occur as diallelic polymorphic forms. Such polymorphic genes have been shown to be associated with the susceptibility to a number of infectious and non-infectious diseases (5).

To find out whether Non-HLA genes are associated in the susceptibility to pulmonary tuberculosis, the following Non-HLA gene polymorphisms, were studied in pulmonary tuberculosis patients (n=202) and control subjects (n=109).

1. Mannose Binding Protein (MBP) genes

Mannose binding protein plays an important role in the host defence against pathogens. Mutations in the genes of mannose binding protein results in low plasma level of this protein which leads to susceptibility to infection. In the present study, wild type and mutant allets of MBP 52, 54 and 57 region of the genes were studied by amplifying the MBP genes of the genomic DNA and the PCR product (bp339) was dot-blotted on nytran membrane and probed with specific oligonucleotide probes and detected using non-radioactive chemiluminiscent system.

2. IL-1 Receptor Antagonist (IL-1RA) gene

IL-1RA is a cytokine which competes for IL-1 binding site and regulates the production of IL-1. 86 base pair tandem repeat mini satellite polymorphism have been identified. This polymorphisms was studied using PCR product and size variants were typed on a 2% agarose gel. Based on the number of 86 bp copies six alleles have been assigned. The percent genotype frequency of these alleles have been studied.

3. Vitamin-D Receptor (VDR) gene

Vitamin-D3 (1,25 dihydroxy vitamin-D3) regulates calcium metabolism. It is an immunoregulatory hormone and activates monocytes and stimulates cell-mediated immune response. The effects are exerted by interaction with the Vitamin-D receptor which is present on monocytes and activated T & B lymphocytes. VDR is a nuclear hormone receptor. Point mutation in the VDR region results in reduced mRNA expression. Single base change polymerphisms of VDR gene were studied.

VDR region of the genomic DNA was amplified, a 361 bp PCR product was dot blotted and detected using specific oligonucleotide probes and chemiluminiscent system.

4. Tumor Necrosis Factor Alpha Gene (TNF α)

Tumor necrosis factor alpha is an inflammatory cytokine mainly produced by monocytes and macrophage. This cytokine plays an important role in pathogenesis of severe infectious disease. Promoter region polymorphism (G-> A mutation at - 308)

affects the regulation of transcriptional start site of TNR alpha gene. Two alletic forms TNF1 (wild) and TNF2 (mutant) have been assigned. TNF2 allele is associate with higher constitutive and inducible levels of transcription than the TNF1 allete. Similarly, another gene polymorphic at 238 promotor region (G-> A mutation) has been identified. For the present study both - 308 and - 238 TNF alpha polymorphisms have been studied.

TNF alpha region of the genome was amplified (PCR product size 693 bp) and polymorphism at - 308 and - 238 have been studied using dot-blot, oligonucleotide probes and chemiluminiscent detection system.

5. *Inducible Nitric Oxide Synthase (iNOS) gene

Nitric oxide has been shown to be microbicidal. Inducible nitric oxide synthase (iNOS) is transcriptionally regulated enzyme that synthesises nitric oxide from L-arginine that has a key role in the pathophysiology of systemic inflammation. CA repeat microsatellite polymorphism has been shown at 313 and 317 region of the iNOS gene. The iNOS gene region was amplified (PCR product : 317 bp) using specific primers with fluorescent lables. CA repeat microsatelite polymorphism was studied by gene scan analysis.

6. *Natural Resistance associated macrophage protein-1(NRAMP-1)

In mice BCG gene has been shown to affect resistance to several intracellular pathogens such as Leishmania parasites (Lsh). Salmonell (Italy) and some strains of Bacille Calmette Guerin (BCG). The equivalence for the BCG gene is the NRAMP-1 gene. The gene encodes for the natural resistance associated macrophage protein (NRAMP) which is involved in macrophage activation. This gene is identified as a candidate for the murine macrophage resistance gene against intracellular pathogens. The human homologue of NRAMP-1 has, CA repeat microsatellite polymorphisms at 199 and 201 region of the human NRAMPM-1 gene. The NRAMP-1 gene was amplified using specific primers with fluorescent labels. The CA repeat polymorphism was studied by gene scan analysis.

*Both iNOS and NRAMP-1 gene polymorphisms were done in collaboration with Richard Bellamy, WTCHG, Oxford, UK.

Results

Genotyping of MPB 52, 54 and 57 variants showed a significant increase of functional mutant homozygotes of the over all MBP genes in PTB patients than control subjects (P=0.008) (Table).

TABLE
PERCENT PHENOTYPE FREQUENCY OF HLA-DR2 AND GENOTYPE FREQUENCY OF FUNCTIONAL MUTANT HOMOZYGOTES OF MANNOSE BINDING PROTEIN GENES OF CONTROL SUBJECTS AND PULMONARY - TB PATIENTS

Candidate Genes	Control Subjects	PTB Patients	P Value	Relative risk/Odds ratio
HLA* HLAW-DR2	29.5 (n-122)	48.8 (n=209)	P<0.001	2.3 RR
Non-HLA* MBP-FMH	1.8 (n=109)	10.9 (n=202)	P=0.008	6.5 OR

* Results taken from Ref.No.4 and Ref.No.7 respectively.

MBP - FMH : Functional Mutant Homozygotes of Mannose Binding Protein genes.

Numbers in the parentheses represent the actual number of control subjects and patients studied.

RR - Relative Risk : OR : Odds ratio.

None of the IL-1RA alleles was associated with pulmonary tuberculosis. However, allele six (single repeat-around 118 bp) was seen in one patient ourr of 202 patients (percent genotye frequency 0.5%) and not in control subjects. So far single repeat has not been reported in other populations.

No association was seen with other gene polymorphisms studied in pulmonary - TB patients and control subjects.

CONCLUSION

The functional mutant homozygotes of the overall mannose binding protein gene variants 52, 54 and 57 are associated with pulmonary tuberculosis.

IL-IRA, VDR, TNF-alpha, iNOS and NRAMP-1 genes are not associated with the susceptibility to pulmonary - TB. The present study suggests that Non-HLA genes are also associated in the susceptibility to PTB, apart from HLA from HLA-DR2.

Identification of new host-genes that are associated with the susceptibility and resistance to *M.tuberculosis* in infection/disease development, may be beneficial and a battery of susceptibility genes for TB may serve as genetic markets to predetermine the development of the disease.

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Recommendations of the Workshop

Recommendations of workshop : Tuberculosis Research : Into the 21st Century

The long-term consequences of the emergence of multi-drug resistant strains of *M.tuberculosis* are largely unknown, but must be a major area of concern. The association between HIV infection and tuberculosis has had major impact on many parts of the world: the impact of HIV, and its consequences for tuberculosis infection in India are currently unclear, but again must be a cause of major concern for the future. Thus we would regard tuberculosis research - from operational studies to fundamental research - as being of the highest priority.

The long traditions of world leadership in TB research in Europe and India and the current strengths of research groups in these countries emphasise the added impact which could be achieved by establishing both multilateral and bilateral funding programmes for collaborations between Indian and European groups.

Areas of scientific priority

1. Fundamental Studies

i. Host-parasite interactions : Studies which aim to understand the interaction between *M.tuberculosis* and host cells (macrophages) at both the cellular and molecular level.

ii. Functional genomics of *M.tuberculosis* : the completion of the *M.tuberculosis* genome project has produced new opportunities for understanding the biology of the bacterium. This information now needs to be utilised to study the function of unknown genes.

iii. Gene regulation : understanding the specialised mechanisms which mycobacteria have evolved to regulate gene expression should give new insights into the evolution of pathogenicity.

iv. Host genetics : with the rapid expansion of the human genome project, it is important to utilise this information to understand the host genetic factors which are involved in susceptibility / resistance to infection. Although it is clear that susceptibility to TB is a polygenic trait, such studies will provide important clues to mechanism of resistance / susceptibility.

Applied Laboratory Research

i. Immunological Studies : Understanding protective immunity and establishing correlates of protection remain a priority for vaccine development.

ii. Animal models : Co-ordination of activities which utilise animal models (e.g. vaccine testing), so that different models can be compared.

iii. Application of molecular methods for the clinical reference laboratory : Methods for rapid diagnosis, investigating variations in strains of *M.tuberculosis*, drug-sensitivity testing, etc. should be evaluated in the clinical reference laboratory setting.

3. Control and Operational Research

i. Studies to investigate different models for effective case finding and treatment monitoring should be carried out.

ii. Studies to investigate the impact of health education strategies are also required.

It was felt that there could be considerable additional benefits from supporting programmes of collaborative research between India and European groups. Such initiatives might take the form of :

1. **Multi-lateral research programmes** : As in the fourth framework, the fifth framework should identify tuberculosis as research priority for the funding of multi-lateral programmes, similar to those projects supported previously by INDO-EC.

2. **Bilateral Research Programmes :** The possibility of funding bilateral collaborative projects between groups working on tuberculosis in India and Europe should be given priority. Such projects might be more focused than those funded under multilateral programmes and should seek to bring together groups with complementary interests to undertake specific research projects.

3. **Short-term interactive exchange** : In order to establish interactions between European and Indian groups with mutual interests in tuberculosis research, priority should be given to establishing short-term interactive visits by scientists. These visits could last between one and three months, and be aimed at establishing initial contact with a view to stimulating a more long-term collaborative proposal.

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